Docket No.: 4614-0160PUS1
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Marc DONATH

Application No.: 10/517,450 | Confirmation No.: 5584

Filed: September 1, 2005 | Art Unit: 1647

For: USE OF AN INTERLEUKIN 1 RECEPTOR ANTAGONIST AND/OR PYRROLIDINEDITHIOCARBAMATE FOR THE TREATMENT OR PROPHYLAXIS OF TYPE 2 DIABETES | Examiner: I. D. Dang

DECLARATION UNDER 37 CFR 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I Dr. Steven E. Kahn, submit this declaration in connection with the prosecution of U.S. Patent Application No. 10/517,450.

1. I am well knowledgeable about the field of diabetes as evidenced by my curriculum vitae, attached hereto as Appendix 1.

2. I understand that during prosecution of U.S. Patent Application No. 10/517,450 the issue has been raised concerning the meaning of the term "insulin diabetes". I also understand that the U.S. Examiner has taken the position that the term "insulin diabetes" in a publication would be understood to "encompass type-II diabetes".

In particular, I understand that the U.S. Patent Examiner has taken the position that the U.S. Patents to Boone et al. and Thompson et al. (attached hereto as Appendix 2 and 3, respectively)

both refer to "insulin diabetes" and that those publications would therefore be referencing both type 1 and type 2 diabetes.

3. I am familiar with both the Boone et al. and Thompson et al. U.S. Patents. Boone et al. at column 1, line 62 refers to "diabetes (e.g., insulin diabetes)" and the Thompson et al. Patent at column 2, line 46 refers to "insulin diabetes".

It is my opinion that the references to "insulin diabetes" in the Thompson et al. and Bonne et al. U.S. Patents would be understood by those skilled in the relevant art as being a shorthand reference to type 1 diabetes and would not be understood to encompass type 2 diabetes.

4. Type 1 diabetes is often referred to as "insulin-dependent diabetes mellitus", or "IDDM". Type 1 diabetes is currently understood to be an autoimmune disease that results in the permanent destruction of the insulin-producing beta cells of the pancreas.

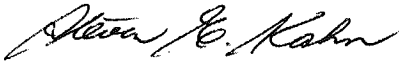
5. On the other hand, type 2 diabetes is often referred to as "non-insulin dependent diabetes mellitus", or "NIDDM". Type 2 diabetes is primarily characterized by the inability of cells to appropriately respond to insulin whereby insulin produced by the pancreas cannot connect with fat and muscle cells to permit glucose inside to thereby produce energy.

6. Thus, publications, such as the Boone et al. and Thompson et al., that use to the term "insulin diabetes" would be understood by those skilled in the field of insulin to refer to type 1 diabetes. Those persons skilled in the field would not understand the term "insulin diabetes" to refer to or encompass the different condition known as type 2 diabetes.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 17 January 2009

By 

Steven E. Kahn

CURRICULUM VITAE

STEVEN EMANUEL KAHN, M.B., Ch.B.

Personal Data:

Place and Date of Birth:	Durban, South Africa; July 28, 1955
Marital Status:	Married (Stephanie Berk); two children
Citizenship:	U.S.A.

Education:

Glenwood High School, Durban, South Africa, 1968-1972
University of Cape Town, South Africa, M.B., Ch.B., 1973-1978

National Service:

Compulsory National Service, South African Medical Services, 1980-1981

Postgraduate Training:

1979:	Intern, Departments of Obstetrics and Gynecology and Medicine, Somerset Hospital, Cape Town, South Africa
1980:	Resident, Department of Obstetrics and Gynecology, 2 Military Hospital, Wynberg, South Africa
1981:	Resident and Coordinator, Department of Obstetrics and Gynecology, 2 Military Hospital, Wynberg, South Africa
1982:	Resident, Division of Endocrinology, Department of Medicine, Groote Schuur Hospital, Cape Town, South Africa
1983:	Research Fellow, Diabetes and Endocrine Research Group, Department of Medicine, University of Cape Town, Cape Town, South Africa
1983-1986:	Resident, Department of Medicine, Albert Einstein Medical Center, Philadelphia, Pennsylvania
1986-1988:	Senior Research Fellow, Division of Metabolism, Endocrinology and Nutrition, Department of Medicine, University of Washington School of Medicine and Veterans Affairs Medical Center, Seattle, Washington

Positions Held:

1988-1991:	Associate Investigator and Staff Physician, Division of Endocrinology and Metabolism, Department of Medicine, Veterans Affairs Medical Center, Seattle, Washington
1988-1992:	Acting Instructor, Division of Metabolism, Endocrinology and Nutrition, Department of Medicine, University of Washington School of Medicine, Seattle, Washington
1991-1995:	Research Associate and Staff Physician, Division of Endocrinology and Metabolism, Department of Medicine, Veterans Affairs Medical Center, Seattle, Washington
1992-1995:	Assistant Professor, Division of Metabolism, Endocrinology and Nutrition, Department of Medicine, University of Washington School of Medicine, Seattle, Washington
1995-2001:	Associate Professor, Division of Metabolism, Endocrinology and Nutrition, Department of Medicine, University of Washington School of Medicine, Seattle, Washington
1995-2001:	Associate Program Director, General Clinical Research Center, Department of Medicine, University of Washington School of Medicine, Seattle, Washington
1995-present:	Associate Director, Diabetes Endocrinology Research Center, Department of Medicine, University of Washington School of Medicine, Seattle, Washington
2001-present:	Professor, Division of Metabolism, Endocrinology and Nutrition, Department of Medicine, University of Washington School of Medicine, Seattle, Washington
2001-present:	Associate Chief of Staff for Research and Development and Staff Physician, Veterans Affairs Puget Sound Health Care System, Seattle, Washington

Honors:

1973-1975:	Amelia Schenkman Scholarship
1984:	Herman Ostrum Memorial Award
1984:	Resident Achievement Award
1988:	Associate Investigator of the Department of Veterans Affairs
1988:	Juvenile Diabetes Foundation Career Development Award
1989:	Dana Foundation Feasibility Award
1991:	Research Associate of the Department of Veterans Affairs
1991:	NIH Clinical Investigator Award
1992-1994:	Diabetes Research Council New Investigator Award
1999:	Pfizer Visiting Professor in Diabetes to Case Western Reserve University
2001:	Novartis Young Investigator in Diabetes Award

2003:	Pfizer Visiting Professor in Diabetes to University of Texas, Houston and Baylor College of Medicine
2004:	Bernard Pimstone Memorial Lecture, Society for Endocrinology, Metabolism and Diabetes of South Africa
2004:	American Diabetes Association Distinguished Clinical Scientist Award
2005:	Robert H. Williams – Rachmiel Levine Award
2006:	McGill Lectureship on Metabolism, McGill University, Montreal
2007:	Banting and Best Lecture, Canadian Diabetes Association

Board Certification:

1978:	Educational Commission for Foreign Medical Graduates (ECFMG)
1983:	FLEX
1986:	American Board of Internal Medicine
1989:	American Board of Internal Medicine (Endocrinology and Metabolism)

Current Licensure:

1979:	South African Medical and Dental Council - Medical Practitioner
1984:	Pennsylvania State Board of Medicine - Medical Practitioner
1986:	Washington State Board of Medicine - Medical Practitioner

Professional Memberships:

American College of Physicians
American Diabetes Association
American Federation for Medical Research
American Society for Clinical Investigation
European Association for the Study of Diabetes
The Endocrine Society
Western Association of Physicians
Western Society for Clinical Investigation

Editorial Responsibilities:

1995-1998:	Editorial Board, The Journal of Clinical Endocrinology and Metabolism
1997-1999:	Editorial Board, Diabetes Care
2001-2004:	Editorial Board, The Journal of Clinical Endocrinology and Metabolism
2005-2007:	Associate Editor, The Journal of Clinical Endocrinology and Metabolism
2005-present:	Editorial Board, Diabetes
2007-present:	Editorial Board, Clinical and Translational Science
2008-present:	Deputy Editor, The Journal of Clinical Endocrinology and Metabolism

Special National Responsibilities:

1994:	Chair, Program Committee for Metabolism, Western Regional Meetings of American Federation for Medical Research, American Society for Clinical Investigation

1994-1996:	Councilor, Western Section, American Federation for Medical Research
1994-1997:	Member and Vice-Chair, Research Grant Review Panel, American Diabetes Association
1996:	Chair, Program Committee for Metabolism, Western Regional Meetings of American Federation for Medical Research, American Society for Clinical Investigation
1996-1998:	Chair-Elect and Chair, Western Section, American Federation for Medical Research and National Councilor, American Federation for Medical Research
1998:	Chair, Program Committee for Metabolism, Western Regional Meetings of American Federation for Medical Research, American Society for Clinical Investigation
2001-2003:	President-Elect and President, Western Society for Clinical Investigation
2002:	Chair, Program Committee for Metabolism, Western Regional Meetings of American Federation for Medical Research, American Society for Clinical Investigation
2001-2002:	Member, Scientific and Medical Meetings Oversight Committee, American Diabetes Association
2002-2004:	Chair, Scientific and Medical Meetings Oversight Committee, American Diabetes Association
2002-2005:	Member, Board of Directors, American Diabetes Association
2004-2006:	Member, Professional Practice Committee, American Diabetes Association
2004-2006:	Member, Task Force on Effective Governance, American Diabetes Association
2005:	Course Director, 52nd American Diabetes Association Postgraduate Course
2005:	Member, Scientific Program Committee, 41st Annual Meeting of the European Association for the Study of Diabetes
2006:	Course Director, 53rd American Diabetes Association Postgraduate Course

Special Local Responsibilities:

1990-2001:	General Clinical Research Center Scientific Advisory Committee, University of Washington
1992-1993:	Scientific Review Committee, VA Puget Sound Health Care System
1993-1998:	Board of Directors and Executive, American Diabetes Association, Washington Affiliate
1999-present:	VA Research and Development Committee, VA Puget Sound Health Care System
2001-present:	Dean's Committee, University of Washington and VA Puget Sound Health Care System
2001-present:	Member, Board of Directors, Seattle Institute for Biomedical and Clinical Research

BIBLIOGRAPHY

Steven Emanuel Kahn, M.B., Ch.B.

Peer Reviewed Publications of Original Work

1. Kahn SE, Miller JL: Rapid resolution of visual field defects and reduction in macroprolactinoma size with bromocriptine therapy: a case report. S Afr Med J 62:696-699; 1982.

2. Kahn SE, Klaff LJ: The importance of metabolic control in diabetes mellitus. S Afr J Cont Med Ed 1:iii 15-23; 1983.

3. Kahn SE, Maxwell JU, Barron JL: Salivary cortisol assessment in the evaluation of hypothalamic-pituitary-adrenal function. S Afr Med J 65:843-846; 1984.

4. Ismail F, Miller JL, Kahn SE, Willcox P: Hypothalamic-pituitary sarcoidosis: a case report and review of the literature. S Afr Med J 67:139-142; 1985.

5. Kahn SE, Goldstein J, Cope C: Low-dose streptokinase therapy for Swan-Ganz catheter-induced thrombosis. Am Heart J 110:891-893; 1985.

6. Kahn SE, Kotler MN, Goldman AP, Ablaza S: Superior vena cava obstruction secondary to acute dissecting aneurysm of the aorta. Am Heart J 111:606-608; 1986.

7. Kochar G, Kahn SE, Kotler MN: Bretylium tosylate and ventricular fibrillation in hypothermia. Ann Int Med 105:624; 1986.

8. Feitelberg SP, Kahn SE, Kotler MN, Cope C, Nakhjavan FK, Lippmann M: Transfemoral embolectomy for massive pulmonary embolus and associated myocardial infarction. Am Heart J 113:819-820; 1987.

9. McCulloch DK, Raghu PK, Johnston C, Klaff LJ, Kahn SE, Beard JC, Ward WK, Benson EA, Koerker DJ, Bergman RN, Palmer JP: Defects in ß-cell function and insulin sensitivity in normoglycemic streptozocin-treated baboons: a model of preclinical insulin-dependent diabetes. J Clin Endocrinol Metab 67:785-792; 1988.

10. Schumer M, Miller-Crain G, Pfeifer MA, The Statil Study Group: Diabetic autonomic neuropathy - part II: coefficient of variation of RR-variation and valsalva maneuver tests. Am J Med 85 (Suppl 5A):144-146; 1988.

11. Kahn SE, Beard JC, Schwartz MW, Ward WK, Ding HL, Bergman RN, Taborsky GJ Jr, Porte D Jr: Increased ß-cell secretory capacity as mechanism for islet adaptation to nicotinic acid-induced insulin resistance. Diabetes 38:562-568; 1989.

12. Porte D Jr, Kahn SE: Hyperproinsulinemia and amyloid in NIDDM: clues to etiology of islet ß-cell dysfunction? Diabetes 38:1333-1336; 1989.

13. McCulloch DK, Klaff LJ, Kahn SE, Schoenfeld SL, Greenbaum CJ, Mauseth R, Benson EA, Nepom GT, Shewey L, Palmer JP: Nonprogression of subclinical ß-cell dysfunction among first-degree relatives of IDDM patients: 5-yr follow-up of the Seattle family study. Diabetes 39:549-556; 1990.

14. Kahn SE, D'Alessio DA, Schwartz MW, Fujimoto WY, Ensinck JW, Taborsky GJ Jr, Porte D Jr: Evidence of cosecretion of islet amyloid polypeptide and insulin by ß-cells. Diabetes 39:634-638; 1990.

15. Saad MF, Kahn SE, Nelson RG, Pettitt DJ, Knowler WC, Schwartz MW, Kowalyk S, Bennett PH, Porte D Jr: Disproportionately elevated proinsulin in Pima Indians with non-insulin-dependent diabetes mellitus. J Clin Endocrinol Metab 70:1247-1253; 1990.

16. Kahn SE, Larson VG, Beard JC, Cain KC, Fellingham GW, Schwartz RS, Veith RC, Stratton JR, Cerqueira MD, Abrass IB: Effect of exercise on insulin action, glucose tolerance and insulin secretion in aging. Am J Physiol 258:E937-E943; 1990.

17. Schwartz MW, Figlewicz DF, Kahn SE, Baskin DG, Greenwood MRC, Porte D Jr: Insulin binding to brain capillaries is reduced in genetically obese, hyperinsulinemic Zucker rats. Peptides 11:467-472; 1990.

18. Kahn SE, Klaff LJ, Schwartz MW, Beard JC, Bergman RN, Taborsky GJ Jr, Porte D Jr: Treatment with a somatostatin analog decreases pancreatic B-cell and whole body sensitivity to glucose. J Clin Endocrinol Metab 71:994-1002; 1990.

19. Schwartz MW, Sipols A, Kahn SE, Lattemann DF, Taborsky GJ Jr, Bergman RN, Woods SC, Porte D Jr: Kinetics and specificity of insulin uptake from plasma into cerebrospinal fluid. Am J Physiol 259:E378-E383; 1990.

20. Schwartz RS, Shuman WP, Bradbury VL, Cain KC, Fellingham GW, Beard JC, Kahn SE, Stratton JR, Cerqueira MD, Abrass IB: Body fat distribution in healthy young and older men. J Gerontol 45:M181-M185; 1990.

21. McCulloch DK, Kahn SE, Schwartz MW, Koerker DJ, Palmer JP: Effect of nicotinic acid-induced insulin resistance on pancreatic B cell function in normal and streptozocin-treated baboons. J Clin Invest 87:1395-1401; 1991.

22. Schwartz RS, Shuman WP, Larson V, Cain KC, Fellingham GW, Beard JC, Kahn SE, Stratton JR, Cerqueira MD, Abrass IB: The effect of intensive endurance exercise training on body fat distribution in young and older men. Metabolism 40:545-551; 1991.

23. Schwartz MW, Marks JL, Sipols AJ, Baskin DG, Woods SC, Kahn SE, Porte D Jr: Central insulin administration reduces neuropeptide Y mRNA expression in the arcuate nucleus of food-deprived lean (Fa/Fa) but not obese (fa/fa) Zucker rats. Endocrinology 128:2645-2647; 1991.

24. Stratton JR, Chandler WL, Schwartz RS, Cerqueira MD, Levy WC, Kahn SE, Larson VG, Cain KC, Beard JC, Abrass IB: Effects of physical conditioning on fibrinolytic variables and fibrinogen in young and old healthy adults. Circulation 83:1692-1697; 1991.

25. McCulloch DK, Koerker DJ, Kahn SE, Bonner-Weir S, Palmer JP: Correlations of in vivo ß-cell function tests with ß-cell mass and pancreatic insulin content in streptozocin-administered baboons. Diabetes 40:673-679; 1991.

26. Schwartz MW, Bergman RN, Kahn SE, Taborsky GJ Jr, Fisher LD, Sipols AJ, Woods SC, Steil GM, Porte D Jr: Evidence for entry of plasma insulin into cerebrospinal fluid through an intermediate compartment in dogs. Quantitative aspects and implications for transport. J Clin Invest 88:1272-1281; 1991.

27. Kahn SE, Fujimoto WY, D'Alessio DA, Ensinck JW, Porte D Jr: Glucose stimulates and potentiates islet amyloid polypeptide secretion by the B-cell. Horm Metab Res 23:577-580; 1991.

28. Kahn SE, McCulloch DK, Schwartz MW, Palmer JP, Porte D Jr: Effect of insulin resistance and hyperglycemia on proinsulin release in a primate model of diabetes mellitus. J Clin Endocrinol Metab 74:192-197; 1992.

29. Kahn SE, Bergman RN, Schwartz MW, Taborsky GJ Jr, Porte D Jr: Short-term hyperglycemia and hyperinsulinemia improve insulin action but do not alter glucose action in normal humans. Am J Physiol 262:E518-E523; 1992.

30. Kahn SE, Larson VG, Schwartz RS, Beard JC, Cain KC, Fellingham GW, Stratton JR, Cerqueira MD, Abrass IB: Exercise training delineates the importance of B-cell dysfunction to the glucose intolerance of human aging. J Clin Endocrinol Metab 74:1336-1342; 1992.

31. Schwartz MW, Sipols AJ, Marks JL, Sanacora G, White JD, Scheurink A, Kahn SE, Baskin DG, Woods SC, Figlewicz DP, Porte D Jr: Inhibition of hypothalamic neuropeptide Y gene expression by insulin. Endocrinology 130:3608-3616; 1992.

32. Schwartz RS, Cain KC, Shuman WP, Larson V, Stratton JR, Beard JC, Kahn SE, Cerqueira MD, Abrass IB: Effect of intensive endurance training on lipoprotein profiles in young and older men. Metabolism 41:649-654; 1992.

33. Chandler WL, Veith RC, Fellingham GW, Levy WC, Schwartz RS, Cerqueira MD, Kahn SE, Larson VG, Cain KC, Beard JC, Abrass IB, Stratton JR: Fibrinolytic response during exercise and epinephrine infusion in the same subjects. J Am Coll Cardiol 19:1412-1420; 1992.

34. Stratton JR, Cerqueira MD, Schwartz RS, Levy WC, Veith RC, Kahn SE, Abrass IB: Differences in cardiovascular responses to isoproterenol in relation to age and exercise training in healthy men. Circulation 86:504-512; 1992.

35. Steil GM, Volund A, Kahn SE, Bergman RN: Reduced sample number for calculation of insulin sensitivity and glucose effectiveness from the minimal model: suitability for use in population studies. Diabetes 42:250-256; 1993.

36. Abbate SL, Fujimoto WY, Brunzell JD, Kahn SE: Effect of heparin on insulin-glucose interactions measured by the minimal model technique: implications for reproducibility using this method. Metabolism 42:353-357; 1993.

37. Neifing JL, Greenbaum CJ, Kahn SE, McCulloch DK, Bärmeier H, Lernmark Å, Palmer JP: Prospective evaluation of beta cell function in insulin autoantibody positive relatives of insulin-dependent diabetic patients. Metabolism 42:482-486; 1993.

38. Kahn SE, Verchere CB, D'Alessio DA, Cook DL, Fujimoto WY: Evidence for selective release of rodent islet amyloid polypeptide through the constitutive secretory pathway. Diabetologia 36:570-573; 1993.

39. Kahn SE, Horber FF, Prigeon RL, Haymond MW, Porte D Jr: Effect of glucocorticoid and growth hormone treatment on proinsulin levels in humans. Diabetes 42:1082-1085; 1993.

40. Baura GD, Foster DM, Porte D Jr, Kahn SE, Bergman RN, Cobelli C, Schwartz MW: Saturable transport of insulin from plasma into the central nervous system of dogs in vivo: a mechanism for regulated insulin delivery to the brain. J Clin Invest 92:1824-1830; 1993.

41. Kahn SE, Prigeon RL, McCulloch DK, Boyko EJ, Bergman RN, Schwartz MW, Neifing JL, Ward WK, Beard JC, Palmer JP, Porte D Jr: Quantification of the relationship between insulin sensitivity and B-cell function in human subjects. Evidence for a hyperbolic function. Diabetes 42:1663-1672; 1993.

42. Prigeon RL, Kahn SE, Porte D Jr: Reliability of error estimates from the minimal model: implications for measurements in physiological studies. Am J Physiol 266:E279-E286; 1994.

43. Kahn SE, Prigeon RL, McCulloch DK, Boyko EJ, Bergman RN, Schwartz MW, Neifing JL, Ward WK, Beard JC, Palmer JP, Porte D Jr: The contribution of insulin dependent and insulin independent glucose uptake to intravenous glucose tolerance in healthy human subjects. Diabetes 43:587-592; 1994.

44. Haymond MW, Horber FF, DeFeo P, Kahn SE, Mauras N: Effect of human growth hormone and insulin-like growth factor 1 on whole-body leucine and estimates of protein metabolism. Horm Res 40:92-94; 1993.

45. D'Alessio DA, Kahn SE, Leusner C, Ensinck JW: Glucagon-like peptide 1 enhances glucose tolerance both by stimulation of insulin release and by increasing insulin-independent glucose disposal. J Clin Invest 93:2263-2266; 1994.

46. Fujimoto WY, Abbate SL, Kahn SE, Hokanson JE, Brunzell JD: The visceral adiposity syndrome in Japanese-American men. Obes Res 2:364-371; 1994.

47. Verchere CB, D'Alessio DA, Palmiter RD, Kahn SE: Transgenic mice overproducing human islet amyloid polypeptide have increased insulin storage and secretion. Diabetologia 37:725-728; 1994.

48. Stone LM, Kahn SE, Deeb SS, Fujimoto WY, Porte D Jr: Glucokinase gene variations in Japanese-Americans with a family history of NIDDM. Diabetes Care 17:1480-1483; 1994.

49. D'Alessio DA, Verchere CB, Kahn SE, Hoagland V, Baskin DG, Palmiter RD, Ensinck JW: Pancreatic expression and secretion of human islet amyloid polypeptide in a transgenic mouse. Diabetes 43:1457-1461; 1994.

50. Kahn SE, Leonetti DL, Prigeon RL, Boyko EJ, Bergstrom RW, Fujimoto WY: Proinsulin as a marker for the development of non-insulin dependent diabetes mellitus in Japanese-American men. Diabetes 44:173-179; 1995.

51. Kahn SE, Leonetti DL, Prigeon RL, Boyko EJ, Bergstrom RW, Fujimoto WY: Relationship of proinsulin and insulin with noninsulin-dependent diabetes mellitus and coronary heart disease in Japanese American men: impact of obesity. J Clin Endocrinol Metab 80:1399-1406; 1995.

52. Schwartz MW, Boyko EJ, Kahn SE, Ravussin E, Bogardus C: Reduced insulin secretion: an independent predictor of weight gain. J Clin Endocrinol Metab 80:1571-1576; 1995.

53. Prigeon RL, Kahn SE, Porte D Jr: Changes in insulin sensitivity, glucose effectiveness, and B-cell function in regularly exercising subjects. Metabolism 44:1259-1263; 1995.

54. Baura GD, Foster DM, Kaiyala K, Porte D Jr, Kahn SE, Schwartz MW: Insulin transport from plasma into the central nervous system is inhibited by dexamethasone in dogs. Diabetes 45:86-90; 1996.

55. Seaquist ER, Kahn SE, Clark PM, Hales CN, Porte D Jr, Robertson RP: Hemipancreatectomy causes hyperproinsulinemia in non-diabetic humans. J Clin Invest 97:455-460; 1996.

56. Prigeon RL, Røder ME, Porte D Jr, Kahn SE: The effect of insulin dose on the measurement of insulin sensitivity by the minimal model technique: Evidence for saturable insulin transport in humans. J Clin Invest 97:501-507; 1996.

57. Stone LM, Kahn SE, Fujimoto WY, Deeb SS, Porte D Jr: A ß-cell glucokinase promoter variant is associated with reduced ß-cell function in Japanese American men. Diabetes 45:422-428; 1996.

58. Verchere CB, D'Alessio DA, Palmiter RD, Weir GC, Bonner-Weir S, Baskin DG, Kahn SE: Islet amyloid formation associated with hyperglycemia in transgenic mice with pancreatic beta cell expression of human islet amyloid polypeptide. Proc Natl Acad Sci USA 93:3492-3496; 1996.

59. Best JD, Kahn SE, Ader M, Watanabe RM, Ni T-C, Bergman RN: Role of glucose effectiveness in the determination of glucose tolerance. Diabetes Care 19:1018-1030; 1996.

60. Prigeon RL, Jacobson RK, Porte D Jr, Kahn SE: Effect of sulfonylurea withdrawal on proinsulin levels, B-cell function, and glucose disposal in subjects with noninsulin-dependent diabetes mellitus. J Clin Endocrinol Metab 81:3295-3298; 1996.

61. Kahn SE, Leonetti DL, Prigeon RL, Bergstrom RW, Fujimoto WY: Proinsulin predicts the development of non-insulin dependent diabetes mellitus in Japanese-American men. Diabetic Med 13:S63-S66; 1996.

62. Greenbaum CJ, Kahn SE, Palmer JP: Nicotinamide's effects on glucose metabolism in subjects at risk for insulin dependent diabetes mellitus. Diabetes 45:1631-1634; 1996.

63. Verchere CB, Paoletta M, Neerman-Arbez M, Rose K, Gingerich RL, Kahn SE, Halban PA: Des-(27-31)C-peptide: a novel secretory product of the rat pancreatic beta cell produced by truncation of proinsulin connecting peptide in secretory granules. J Biol Chem 271:27475-27481; 1996.

64. Verchere CB, D'Alessio DA, Wang S, Andrikopoulos S, Kahn SE: Transgenic overproduction of islet amyloid polypeptide (amylin) is not sufficient for islet amyloid formation. Horm Metab Res 29:311-316; 1997.

65. Schwartz MW, Prigeon RL, Kahn SE, Nicolson M, Moore J, Morawiecki A, Boyko EJ, Porte D Jr: Evidence that plasma leptin and insulin levels are associated with body adiposity via different mechanisms. Diabetes Care 20:1476-1481; 1997.

66. Verchere CB, D'Alessio DA, Andrikopoulos S, Kahn SE: A transgenic mouse model of pancreatic islet amyloid formation. Comp Path Bull 30:4-5; 1997.

67. Kahn SE, Halban PA: Release of incompletely processed proinsulin is the cause of the disproportionate proinsulinemia of NIDDM. Diabetes 46:1725-1732; 1997.

68. Ensinck JW, Vogel RE, Laschansky EC, Koerker D, Prigeon RL, Kahn SE, D'Alessio DA: Endogenous somatostatin-28 modulates of postprandial insulin secretion: immunoneutralization studies in baboons. J Clin Invest 100:2295-2302; 1997.

69. Røder ME, Porte D Jr, Kahn SE: Disproportionately elevated proinsulin levels reflect the degree of impaired B-cell secretory capacity in patients with non-insulin dependent diabetes mellitus. J Clin Endocrinol Metab 83:604-608; 1998.

70. Prigeon RL, Røder ME, Kahn SE, Porte D Jr: Effects of troglitazone on proinsulin release and other factors related to glucose tolerance in NIDDM. J Clin Endocrinol Metab 83:819-823; 1998.

71. Kahn SE, Verchere CB, Andrikopoulos S, Asberry PJ, Leonetti DL, Wahl PW, Boyko EJ, Schwartz RS, Newell-Morris L, Fujimoto WY: Reduced amylin release is a characteristic of impaired glucose tolerance and type 2 diabetes mellitus in Japanese Americans. Diabetes 47:640-645; 1998.

72. Dechenes CJ, Verchere CB, Andrikopoulos S, Kahn SE: Human aging is associated with parallel reductions in insulin and amylin release. Am J Physiol 275:E785-E791; 1998.

73. Greenbaum CJ, Sears KL, Kahn SE, Palmer JP: The relationship of β-cell function and autoantibodies to progression and non-progression of subclinical type 1 diabetes. Diabetes 48:170-175; 1999.

74. Kahn SE, Andrikopoulos S, Verchere CB: Islet amyloid: a long recognized but under appreciated pathology of type 2 diabetes mellitus. Diabetes; 48:241-253; 1999.

75. Elbein SC, Hasstedt SJ, Wegner K, Kahn SE: Heritability of pancreatic ß-cell function among nondiabetic members of Caucasian familial type 2 diabetic kindreds. J Clin Endocrinol Metab 84:1398-1403; 1999.

76. Andrikopoulos S, Verchere CB, Teague JC, Howell WM, Fujimoto WY, Wight TN, Kahn SE: Two novel immortal pancreatic ß-cell lines expressing and secreting human islet amyloid polypeptide do not spontaneously develop islet amyloid. Diabetes 48:1962-1970; 1999.

77. Kaiyala KJ, Prigeon RL, Kahn SE, Woods SC, Porte D Jr, Schwartz MW: Reduced ß-cell function contributes to impaired glucose tolerance in dogs made obese by high-fat feeding. Am J Physiol 277:E659-E667; 1999.

78. Elbein SC, Wegner K, Kahn SE: Reduced ß-cell compensation to the insulin resistance associated with obesity in members of Caucasian familial type 2 diabetic kindreds. Diabetes Care 23:221-227; 2000.

79. Purnell JQ, Kahn SE, Albers JJ, Nevin DN, Brunzell JD, Schwartz RS: Effect of weight loss with reduction of intra-abdominal fat on lipid metabolism in older men. J Clin Endocrinol Metab 85:977-982; 2000.

80. Røder ME, Schwartz RS, Prigeon RL, Kahn SE: Reduced pancreatic B-cell compensation to the insulin resistance of aging: impact and implications for proinsulin and insulin levels. J Clin Endocrinol Metab, 85:2275-2280; 2000.

81. Verchere CB, D'Alessio DA, Prigeon RL, Hull RL, Kahn SE: The constitutive secretory pathway is a major route for islet amyloid polypeptide secretion in neonatal but not adult rat islet cells. Diabetes 49:1477-1484; 2000.

82. Kaiyala KJ, Prigeon RL, Kahn SE, Woods SC, Schwartz MW: Obesity induced by a high fat diet is associated with reduced brain insulin transport in dogs. Diabetes 49:1525-1533; 2000.

83. Kahn SE, Andrikopoulos S, Verchere CB, Wang F, Hull RL, Vidal J: Oophorectomy promotes islet amyloid formation in a transgenic mouse model of type 2 diabetes. Diabetologia, 43:1309-1312; 2000.

84. Andrikopoulos S, Verchere CB, Terauchi K, Kadowaki T, Kahn SE: ß-cell glucokinase deficiency and hyperglycemia are associated with reduced islet amyloid deposition in a mouse model of type 2 diabetes. Diabetes 49:2056-2062; 2000.

85. Liao D, Shofer JB, Boyko EJ, McNeely MJ, Leonetti DL, Kahn SE, Fujimoto WY: Abnormal glucose tolerance and increased risk for cardiovascular disease in Japanese-Americans with normal fasting glucose. Diabetes Care 24:39-44; 2001.

86. Purnell JQ, Kahn SE, Schwartz RS, Brunzell JD: Relationship of insulin sensitivity and apoB levels to intra-abdominal fat in subjects with familial combined hyperlipidemia. Arterioscler Thromb Vasc Biol 21:567-572; 2001.

87. Kahn SE: The importance of ß-cell failure in the development and progression of type 2 diabetes. J Clin Endocrinol Metab 86:4047-4058; 2001.

88. Halban PA, Kahn SE, Lernmark Å, Rhodes CJ: Gene and cell-replacement therapy in the treatment of type 1 diabetes: how high must the standards be set? Diabetes 50:2181-2191; 2001.

89. Wang F, Hull RL, Vidal J, Cnop M, Kahn SE: Islet amyloid develops diffusely throughout the pancreas before becoming severe and replacing endocrine cells. Diabetes 50:2514-2520; 2001.

90. Kahn SE, Montgomery B, Howell WM, Ligueros-Saylan M, Hsu C-H, Devineni D, McLeod JF, Horowitz AD, Foley JE: Importance of early phase insulin secretion to intravenous glucose tolerance in subjects with type 2 diabetes mellitus. J Clin Endocrinol Metab 86:5824-5829; 2001.

91. Knowles NG, Landchild MA, Fujimoto WY, Kahn SE: Insulin and amylin release are both diminished in first-degree relatives of subjects with type 2 diabetes mellitus. Diabetes Care 25:292-297; 2002.

92. Cnop M, Landchild M, Vidal J, Havel PJ, Knowles NG, Carr DR, Wang F, Hull RL, Boyko EJ, Retzlaff BM, Walden CE, Knopp RH, Kahn SE: The concurrent accumulation of intra-abdominal and subcutaneous fat explains the association between insulin resistance and plasma leptin concentrations: distinct metabolic effects of two fat compartments. Diabetes 51:1005-1015; 2002.

93. Liao D, Asberry PJ, Shofer JB, Callahan H, Matthys C, Boyko EJ, Leonetti D, Kahn SE, Austin M, Newell L, Schwartz RS, Fujimoto WY: Improvement of BMI, body composition, and body fat distribution with lifestyle modification in Japanese Americans with impaired glucose tolerance. Diabetes Care 25:1504-1510; 2002.

94. Paoletta M, Kahn SE, Halban PA: Truncated (des-[27-31]) C-peptide is not a major secretory product of human islets. Diabetologia 45:1523-1527; 2002.

95. Sweet IR, Khalil G, Wallen AR, Steedman M, Schenkman KA, Reems JA, Kahn SE, Callis JB: Continuous measurement of oxygen consumption by pancreatic islets. Diabetes Technol Ther 4:661-672; 2002.

96. Nieves DJ, Cnop M, Retzlaff BM, Walden CE, Brunzell JD, Knopp RH, Kahn SE: The atherogenic lipoprotein profile associated with obesity and insulin resistance is largely attributable to intra-abdominal fat. Diabetes 52:172-179; 2003.

97. Kahn SE: The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of type 2 diabetes. Diabetologia 46:3-19; 2003.

98. Vidal J, Verchere CB, Andrikopoulos S, Wang F, Hull RL, Cnop M, Olin KL, LeBoeuf RC, O'Brien KD, Chait A, Kahn SE: The effect of apolipoprotein E deficiency on islet amyloid deposition in human islet amyloid polypeptide transgenic mice. Diabetologia 46:71-79; 2003.

99. Hull RL, Andrikopoulos S, Verchere CB, Vidal J, Wang F, Cnop M, Prigeon RL, Kahn SE: Increased dietary fat promotes islet amyloid formation and ß-cell secretory dysfunction. Diabetes 52:372-379; 2003.

100. Hayashi T, Boyko EJ, Leonetti DL, McNeely MJ, Newell-Morris L, Kahn SE, Fujimoto WY: Visceral adiposity and the risk of impaired glucose tolerance: a prospective study among Japanese Americans. Diabetes Care 26:650-655; 2003.

101. McNeely MJ, Boyko EJ, Leonetti DL, Kahn SE, Fujimoto WY: Comparison of a clinical model, the oral glucose tolerance test, and fasting glucose for prediction of type 2 diabetes risk in Japanese Americans. Diabetes Care 26:758-763; 2003.

102. Potter-Perigo S, Hull RL, Tsoi C, Braun KR, Andrikopoulos S, Teague J, Verchere CB, Kahn SE, Wight TN: Proteoglycans synthesized and secreted by pancreatic islet ß-cells bind human amylin. Arch Biochem Biophys 413:182-190; 2003.

103. Cnop M, Havel PJ, Utzschneider KM, Carr DB, Sinha MK, Boyko EJ, Retzlaff BM, Knopp RH, Brunzell JD, Kahn SE: Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. Diabetologia 46:459-469; 2003.

104. Knopp RH, Retzlaff B, Fish B, Walden C, Wallick S, Anderson M, Aikawa K, Kahn SE: Effects of insulin resistance and obesity on lipoproteins and sensitivity to egg feeding. Arterioscler Thromb Vasc Biol 23:1437-1443; 2003.

105. Murdoch SJ, Kahn SE, Albers JJ, Brunzell JD, Purnell JQ: PTLP activity decreases with weight loss: changes in PLTP are associated with changes in subcutaneous fat and FFA but not IAF or insulin sensitivity. J Lipid Res 44:1705-1712; 2003.

106. Hayashi T, Boyko EJ, Leonetti DL, McNeely MJ, Newell-Morris L, Kahn SE, Fujimoto WY: Visceral adiposity and the prevalence of hypertension in Japanese Americans. Circulation 108:1718-1723; 2003.

107. Andrikopoulos S, Hull RL, Verchere CB, Wang F, Wilbur SM, Wight TN, Marzban L, Kahn SE: Extended life span is associated with insulin resistance in a transgenic mouse model of insulinoma secreting human islet amyloid polypeptide. Am J Physiol 286:E418-E424; 2004.

108. Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA, Scherer PE: Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. J Biol Chem 279:12152-12162; 2004.

109. Kahn SE: Engineering a new ß-cell: a critical venture requiring special attention to constantly changing physiological needs. Semin Cell Dev Biol 15:359-370; 2004.

110. Utzschneider KM, Carr DB, Barsness SM, Kahn SE, Schwartz RS: Diet-induced weight loss is associated with an improvement in ß-cell function in older men. J Clin Endocrinol Metab 89:2704-2710; 2004.

111. Hayashi T, Boyko EJ, Leonetti DL, McNeely MJ, Newell-Morris L, Kahn SE, Fujimoto WY: Visceral adiposity is an independent predictor of incident hypertension in Japanese Americans. Ann Intern Med, 140:992-1000; 2004.

112. Carr DB, Utzschneider KM, Hull RL, Kodama K, Retzlaff BM, Brunzell JD, Shofer JB, Fish BE, Knopp RH, Kahn SE: Intra-abdominal fat is a major determinant of the National Cholesterol Education Program Adult Treatment Panel III criteria for the metabolic syndrome. Diabetes 53:2087-2094; 2004.

113. Hull RL, Westermark GT, Westermark P, Kahn SE: Islet amyloid: a critical entity in the pathogenesis of type 2 diabetes. J Clin Endocrinol Metab 89:3629-3643; 2004.

114. Utzschneider KM, Carr DB, Hull RL, Kodama K, Shofer JB, Retzlaff BM, Knopp RH, Kahn SE: Impact of intra-abdominal fat and age on insulin sensitivity and ß-cell function. Diabetes 53:2867-2872; 2004.

115. Ioannou GN, Weiss NS, Boyko EJ, Kowdley KV, Kahn SE, Carithers RL, Dominitz JA: Is central obesity associated with cirrhosis-related death or hospitalization? A population-based, cohort study. Clin Gastroenterol Hepatol 3:67-74; 2005.

116. Carr DB, Utzschneider KM, Boyko EJ, Asberry PJ, Hull RL, Kodama K, Callahan HS, Matthys CC, Leonetti DL, Schwartz RS, Kahn SE, Fujimoto WY: A reduced-fat diet and aerobic exercise in Japanese Americans with impaired glucose tolerance decreases intra-abdominal fat and improve insulin sensitivity but not β-cell function. Diabetes 54:340-347; 2005.

117. Ioannou GN, Weiss NS, Boyko EJ, Kahn SE, Lee SP: Contribution of metabolic factors to alanine aminotransferase activity in persons with other causes of liver disease. Gastroenterology 128:627-635; 2005.

118. Tong J, Fujimoto WY, Kahn SE, Weigle DS, McNeely MJ, Leonetti DL, Shofer JB, Boyko EJ: Insulin, C-peptide, and leptin concentrations predict increased visceral adiposity at 5- and 10 year follow-ups in nondiabetic Japanese Americans. Diabetes 54:985–990; 2005.

119. Retnakaran R, Hanley AJG, Raif N, Hirning CR, Connelly PW, Sermer M, Kahn SE, Zinman B: Adiponectin and beta cell dysfunction in gestational diabetes: pathophysiological implications. Diabetologia 48:993-1001; 2005.

120. Tannock LR, O'Brien KD, Knopp RH, Retzlaff BM, Fish B, Wener MH, Kahn SE, Chait A: Cholesterol feeding increases C-reactive protein and serum amyloid A levels in lean insulin-sensitive subjects. Circulation 111:3058-3062; 2005.

121. Hull RL, Shen Z-P, Watts MR, Kodama K, Carr DB, Utzschneider KM, Zraika S, Wang F, Kahn SE: Long-term treatment with rosiglitazone and metformin reduces the extent of, but does not prevent, islet amyloid deposition in mice expressing the gene for human islet amyloid polypeptide. Diabetes 54:2235-2244; 2005.

122. Hull RL, Kodama K, Utzschneider KM, Carr DB, Prigeon RL, Kahn SE: Dietary-fat-induced obesity in mice results in beta cell hyperplasia but not increased insulin release: evidence for specificity of impaired beta cell adaptation. Diabetologia 48:1350-1358; 2005.

123. Hull RL, Watts MR, Kodama K, Shen Z, Utzschneider KM, Carr DB, Vidal J, Kahn SE: Genetic background determines the extent of islet amyloid formation in human islet amyloid polypeptide transgenic mice. Am J Physiol 289:E703-E709; 2005.

124. Andrikopoulos S, Massa CM, Aston-Mourney K, Funkat A, Fam BC, Hull RL, Kahn SE, Proietto J: Differential effect of inbred mouse strain (C57BL/6, DBA/2, 129T2) on insulin secretory function in response to a high fat diet. J Endocrinology 187:45-53; 2005.

125. Rasouli N, Hale T, Kahn SE, Spencer HJ, Elbein SC: Effects of short-term experimental insulin resistance and family history of diabetes on pancreatic ß-cell function in nondiabetic individuals. J Clin Endocrinol Metab 90:5825-5833; 2005.

126. Watson GS, Cholerton BA, Reger MA, Baker LD, Plymate SR, Asthana S, Fishel MA, Kulstad JJ, Green PS, Cook DG, Kahn SE, Keeling ML, Craft S: Preserved cognition in patients with early Alzheimer disease and amnestic mild cognitive impairment during treatment with rosiglitazone: a preliminary study. Am J Geriatr Pysch 13:950-958; 2005.

127. Utzschneider KM, Carr DB, Tong J, Wallace TM, Hull RL, Zraika S, Xiao Q, Mistry JS, Retzlaff BM, Knopp RH, Kahn SE: Resistin is not associated with insulin sensitivity or the metabolic syndrome in humans. Diabetologia 48:2330-2333; 2005.

128. Watson GS, Reger MA, Baker LD, McNeely MJ, Fujimoto WY, Kahn SE, Boyko EJ, Leonetti DL, Craft S: Effects of exercise and nutrition on memory in Japanese Americans with impaired glucose tolerance. Diabetes Care 29:135-136; 2006.

129. Utzschneider KM, Prigeon RL, Carr DB, Hull RL, Tong J, Shofer JB, Retzlaff BM, Knopp RH, Kahn SE: Impact of differences in fasting glucose and glucose tolerance on the hyperbolic relationship between insulin sensitivity and insulin responses. Diabetes Care 29:356-362; 2006.

130. Krudys KM, Kahn SE, Vicini P: Population approaches to estimate minimal model indexes of insulin sensitivity and glucose effectiveness using full and reduced sampling schedules. Am J Physiol 291:E716-E723; 2006.

131. Utzschneider KM, Kahn SE: The role of insulin resistance in nonalcoholic fatty liver disease. J Clin Endocrinol Metab 91:4753-4761; 2006.

132. Kahn SE, Hull RL, Utzschneider KM: Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 444:840-846; 2006.

133. Hayashi T, Boyko EJ, McNeely MJ, Leonetti DL, Kahn SE, Fujimoto WY: Minimum waist and visceral fat values for identifying Japanese Americans at risk for the metabolic syndrome. Diabetes Care 30:120-127; 2007.

134. Tong J, Utzschneider KM, Carr DB, Zraika S, Udayasankar J, Gerchman F, Knopp RH, Kahn SE: Plasma pancreatic polypeptide levels are associated with differences in body fat distribution in human subjects. Diabetologia 50:439-442; 2007.

135. Zraika S, Hull RL, Udayasankar J, Clark A, Utzschneider KM, Tong J, Gerchman F, Kahn SE: Identification of the amyloid-degrading enzyme neprilysin in mouse islets and potential role in islet amyloidogenesis. Diabetes 56:304-310; 2007.

136. Zraika S, Hull RL, Udayasankar J, Utzschneider KM, Tong J, Gerchman F, Kahn SE: Glucose- and time-dependence of islet amyloid formation *in vitro*. Biochem Biophys Res Commun 354:234-239; 2007.

137. Cnop M, Vidal J, Hull RL, Utzschneider KM, Carr DB, Schraw T, Scherer PE, Boyko EJ, Fujimoto WY, Kahn SE: Progressive loss of ß-cell function leads to worsening glucose tolerance in first-degree relatives of subjects with type 2 diabetes. Diabetes Care 30:677-682; 2007.

138. Gerchman F, Tong J, Utzschneider KM, Hull RL, Zraika S, Udayasankar J, McNeely MJ, Andress DL, Leonetti DL, Boyko EJ, Fujimoto WY, Kahn SE: Superiority of the Modification of Diet in Renal Disease equation over the Cockcroft-Gault equation in screening for impaired kidney function in Japanese Americans. Diabetes Res Clin Pract 77:320-326; 2007.

139. Tong J, Boyko EJ, Utzschneider KM, McNeely MJ, Hayashi T, Carr DB, Wallace TM, Zraika S, Gerchman F, Leonetti DL, Fujimoto WY, Kahn SE: Intra-abdominal fat accumulation predicts the future development of the metabolic syndrome in Japanese Americans. Diabetologia 50:1156-1160; 2007.

140. Wallace TM, Utzschneider KM, Tong J, Carr DB, Zraika S, Bankson DD, Knopp RH, Kahn SE: Relationship of liver enzymes to insulin sensitivity and intra-abdominal fat. Diabetes Care 30:2673-2678; 2007.

141. Hull RL, Zraika S, Udayasankar J, Kisilevsky R, Szarek WA, Wight TN, Kahn SE: Inhibition of glycosaminoglycan synthesis and protein glycosylation with WAS-406 and azaserine result in reduced islet amyloid formation in vitro. Am J Physiol 293:1586-1593; 2007.

142. Utzschneider KM, Prigeon RL, Tong J, Gerchman F, Carr DB, Zraika S, Udayasankar J, Montgomery B, Mari A, Kahn SE: Within-subject variability of measures of beta cell function derived from a 2 h OGTT: implications for research studies. Diabetologia 50:2516-2525; 2007.

143. Utzschneider KM, Tong J, Montgomery B, Udayasankar J, Gerchman F, Marcovina SM, Watson CE, Ligueros-Saylan MA, Foley JE, Holst JJ, Deacon CF, Kahn SE: The dipeptidyl peptidase-4 inhibitor vildagliptin improves ß-cell function and insulin sensitivity in subjects with impaired fasting glucose. Diabetes Care 31:108-113; 2008.

144. Kratz M, Purnell JQ, Breen PA, Thomas KK, Utzschneider KM, Carr DB, Kahn SE, Hughes JP, Rutledge EA, Van Yserloo B, Yukawa M, Weigle DS: Reduced adipogenic gene expression in thigh adipose tissue precedes human immunodeficiency virus-associated lipoatrophy. J Clin Endocrinol Metab 93:959-966; 2008.

145. Carr DB, Newton KM, Utzschneider KM, Tong J, Gerchman F, Kahn SE, Heckbert SR: Modestly elevated glucose levels during pregnancy are associated with a higher risk of future diabetes amongst women without gestational diabetes mellitus. Diabetes Care 31:1037-1039; 2008.

146. Hayashi T, Boyko EJ, McNeely MJ, Leonetti DL, Kahn SE, Fujimoto WY: Visceral adiposity, not abdominal subcutaneous fat area, is associated with an increase in future insulin resistance in Japanese Americans. Diabetes 57:1269-1275; 2008.

147. Udayasankar J, Kodama K, Hull RL, Zraika S, Aston-Mourney K, Subramanian SL, Tong J, Faulenbach MV, Vidal J, Kahn SE: Amyloid formation results in recurrence of hyperglycemia following transplantation of human IAPP transgenic mouse islets. Diabetologia 52:145-153; 2009.

148. Carr DB, Newton KM, Utzschneider KM, Tong J, Gerchman F, Kahn SE, Easterling TR, Heckbert SR: Preeclampsia and risk of developing subsequent diabetes. Hypertens Pregnancy, in press.

149. Utzschneider KM, Prigeon RL, Faulenbach MV, Tong J, Carr DB, Boyko EJ, Leonetti DL, McNeely MJ, Fujimoto WY, Kahn SE: An oral disposition index predicts the development of future diabetes above and beyond fasting and 2-hour glucose levels. Diabetes Care, in press.

150. Purnell JQ, Kahn SE, Samuels MH, Brandon D, Loriaux DL, Brunzell JD: Enhanced cortisol production rates, free cortisol, and 11 beta HSD-1 expression correlate with visceral fat and insulin resistance in men: effect of weight loss. Am J Physiol, in press.

151. Zraika S, Hull RL, Udayasankar J, Aston-Mourney KL, Subramanian SL, Kisilevsky R, Szarek WA, Kahn SE: Oxidative stress is induced by islet amyloid formation and time-dependently mediates amyloid-induced beta cell apoptosis. Diabetologia, in press.

Invited Editorials and Reviews

1. Kahn SE, Klaff LJ: The importance of metabolic control in diabetes mellitus. S Afr J Cont Med Ed 1:iii 15-23; 1983.

2. Kahn SE, Porte D Jr: Pathophysiology of non-insulin-dependent diabetes mellitus: implications for treatment. Saudi Med J 9:580-587; 1988.

3. Kahn SE, Porte D Jr: Islet dysfunction in non-insulin-dependent diabetes mellitus. Am J Med 85 (Suppl 5A):4-8; 1988.

4. Porte D Jr, Kahn SE: Pathophysiology of non-insulin-dependent diabetes mellitus: implications for treatment. Il Diabete 1:25-30; 1989.

5. Porte D Jr, Kahn SE: What geriatricians should know about diabetes mellitus. Diabetes Care 13 (Suppl 2):47-54; 1990.

6. Kahn SE, Schwartz RS, Porte D Jr, Abrass IB: The glucose intolerance of aging. Implications for intervention. Hosp Pract 26:29-38; 1991.

7. Porte D Jr, Kahn SE: Mechanisms for hyperglycemia in type II diabetes mellitus: therapeutic implications for sulfonylurea treatment - an update. Am J Med 90 (Suppl 6A):8S-14S; 1991.

8. Porte D Jr, Kahn SE: The key role of islet dysfunction in type II diabetes mellitus. Clin Invest Med 18:247-254; 1995.

9. Kahn SE: Regulation of B-cell function in vivo: from health to disease. Diabetes Rev 4:372-389;1996.

10. Schwartz MW, Kahn SE: Insulin resistance and obesity. Nature 402:860-861; 1999.

11. Kahn SE: The importance of the ß-cell in the pathogenesis of type 2 diabetes mellitus. Am J Med 108 (Suppl 6A):2S-8S; 2000.

12. Fujimoto WY, Bergstrom RW, Boyko EJ, Chen K, Kahn SE, Leonetti DL, McNeely MJ, Newell LL, Shofer JB, Wahl PW: Type 2 diabetes and the metabolic syndrome in Japanese Americans. Diabetes Res Clin Pract 50 (Suppl 2): S73-S76; 2000.

13. Kahn, SE, Prigeon RL, Schwartz RS, Fujimoto WY, Knopp RH, Brunzell JD, Porte D Jr: Obesity, body fat distribution, insulin sensitivity and islet ß-cell function as explanations for metabolic diversity. J Nutr 131:354S-360S; 2001.

14. Porte D Jr, Kahn SE: ß-cell dysfunction and failure in type 2 diabetes: potential mechanisms. Diabetes 50 (Suppl 1):S160-S163; 2001.

15. Hull RL, Verchere CB, Andrikopoulos S, Wang F, Vidal J, Kahn SE: Oophorectomy promotes islet amyloid formation in human islet amyloid polypeptide transgenic mice. Diabetes 50 (Suppl 1):S184-S185; 2001.

16. Fujimoto WY, Bergstrom RW, Boyko EJ, Chen K-W, Kahn SE, Leonetti DL, McNeely MJ, Newell LL, Shofer JB, Tsunehara CH, Wahl PW: Preventing diabetes – applying pathophysiologic and epidemiologic evidence. Br J Nutr 84 (Suppl 2):S173-S176; 2001.

17. Kahn SE: Beta cell failure: causes and consequences. Int J Clin Pract (Suppl 123):13-18; 2001.

18. Kahn SE: Insulin resistance and beta cell dysfunction: a dual therapeutic approach. Eur J Clin Invest 32 (Suppl 3):1-2; 2002.

19. Bergman RN, Finegood DT, Kahn SE: The evolution of beta-cell dysfunction and insulin resistance in type 2 diabetes. Eur J Clin Invest 32 (Suppl 3):35-45; 2002.

20. Kahn SE: The importance of ß-cell dysfunction in the pathogenesis of type 2 diabetes mellitus. Harrison's Online, McGraw Hill, 2002. (Accessed October 25, 2002, at http://www.harrisonsonline.com/server-java/Arknoid/amed/harrisons/ex_editorials/edl3353_p01.html)

21. Knopp RH, Retzlaff B, Aikawa K, Kahn SE: Management of patients with diabetic hyperlipidemia. Am J Cardiol 91 (Suppl):24E-28E; 2003.

22. Fonseca VA, Kahn SE: Of Hopes and DREAMS: the quest to prevent type 2 diabetes. J Clin Endocrinol Metab 91:4762-4763; 2006.

23. Kahn SE, Zinman B: Recent long-term clinical studies support an enhanced role for thiazolidinediones in the management of type 2 diabetes. Diabetes Care 30:1672-1676; 2007.

24. Kahn SE, Carr DB, Faulenbach MV, Utzschneider KM: An examination of ß-cell function measures and their potential use for estimating ß-cell mass. Diabetes Obes Metab 10 (Suppl 4):63-76; 2008.

25. Kahn SE, Cefalu WT: Glucose control, macro- and microvascular disease, and the Food and Drug Administration: Let's keep our eye on the ball. J Clin Endocrinol Metab 93:3727-3729; 2008.

Book Chapters and Other Publications

1. Davidson JS, Kahn SE, Rayner B, Levitt NS, Ames FR, Disler PB, Bonafede P: The medical care of prisoners and detainees (letter). S Afr Med J 64:116; 1983.

2. Kahn SE, Porte D Jr: Pathophysiology of islet dysfunction in non-insulin-dependent (type II) diabetes mellitus. In: Diabetes 1988. Proceedings of the 13th Int. Diabetes Federation, Sydney, Australia, RG Larkins, PZ Zimmet, and DJ Chisholm, eds., Excerpta Medica, Amsterdam, ICS#800, 1989, p 479-483.

3. Kahn SE, Porte D Jr: Pathophysiology of islet dysfunction in non-insulin-dependent (type II) diabetes mellitus. In: Non-Insulin-Dependent Diabetes Mellitus. D Cameron, S Colagiuri, L Heding, C Kühl, A Ma, and R Mortimer, eds., Excerpta Medica, Hong Kong, 1989, p 10-14.

4. Kahn SE, Porte D Jr: Pathophysiology of type II diabetes mellitus: implications for treatment. In: Diabetes Mellitus: Theory and Practice, H Rifkin, and D Porte Jr, eds., Elsevier, New York, 1990, p 436-456.

5. Porte D Jr, Kahn SE: Hyperproinsulinemia and amyloid in type 2 diabetes. In: Frontiers in Diabetes Research: Lessons from Animal Diabetes III, E Shafrir, ed., Smith-Gordon, London, 1991, p 507-511.

6. Wallum BJ, Kahn SE, McCulloch DK, Porte D Jr: Insulin secretion in the normal and diabetic human. In: The International Textbook of Diabetes Mellitus, KGMM Alberti, RA DeFronzo, H Keen, and P Zimmet, eds., John Wiley and Sons, England, 1992, p 285-301.

7. Porte D Jr, Kahn SE: Hyperproinsulinemia and amyloid in type II diabetes. In: Progress in Endocrinology. Proceedings of the 9th Int. Congress of Endocrinology, Nice, France, R Mornex, C Jaffiol, and J Leclere, eds., Parthenon Publishing, England, 1993, p 800-803.

8. Kahn SE, Prigeon RL, Porte D Jr: Insulin and proinsulin in normal and diabetic humans. In: Insulin Secretion and Pancreatic B-cell Research, PR Flatt and S Lenzen, eds., Smith-Gordon, London, 1994, p 391-401.

9. Fujimoto WY, Kahn SE, Brunzell JB: Metabolic basis for coronary heart disease risk in central obesity and glucose intolerance. In: Multiple Risk Factors in Cardiovascular Disease, A Yamamoto, ed., Churchill Livingstone, Tokyo, 1994, p 189-192.

10. Verchere CB, D'Alessio DA, Kahn SE: Consequences of human islet amyloid polypeptide expression in transgenic mice. In: Lessons from Animal Diabetes VI: 75th Anniversary of the Insulin Discovery, E Shafrir, ed., Birkhäuser, Boston, 1996, p 131-148.

11. Kahn SE, Porte D Jr: Pathophysiology of type II diabetes mellitus. In: Diabetes Mellitus, 5th edition, D Porte Jr and RS Sherwin, eds., Elsevier, New York, 1996, p 487-512.

12. Kahn SE: Assessment of B-cell function in humans: approach and interpretation. In: Clinical Research in Diabetes and Obesity, B Draznin and RA Rizza, eds., Humana Press, Totawa NJ, 1997, p 3-22.

13. Kahn SE, McCulloch DK, Porte D Jr: Insulin secretion in the normal and diabetic human. In: The International Textbook of Diabetes Mellitus, 2nd edition, KGMM Alberti, RA DeFronzo, H Keen, and P Zimmet, eds., John Wiley and Sons, England, 1997, p 337-353.

14. Kahn SE, Porte D Jr: ß-cell dysfunction in type 2 diabetes: pathophysiologic and genetic bases. In: The Metabolic and Molecular Bases of Inherited Disease, 8th edition, CR Scriver, AL Beaudet, WS Sly, D Valle, B Childs, KW Kinzler and B Vogelstein, eds., McGraw-Hill, New York, 2001, p 1407-1431.

15. Vidal J, Kahn, SE: Regulation of insulin secretion in vivo. In: Genetics of Diabetes Mellitus, WL Lowe Jr, ed., Kluwer, Boston, 2001, p 109-131.

16. Kahn SE, Porte D Jr: The pathophysiology and genetics of type 2 diabetes mellitus. In: Diabetes Mellitus, 6th edition, D Porte Jr, RS Sherwin and AD Baron, eds., McGraw-Hill, New York, 2002, p 331-365.

17. Hull RL, Kahn SE: Islet amyloid in type 2 diabetes. In: Diabetes Mellitus: A Fundamental and Clinical Text, 3rd edition, D LeRoith, SI Taylor, and JM Olefsky, eds., Lippincott, Williams and Wilkins, Philadelphia, 2004, p 151-161.

18. Utzschneider KM, Porte D Jr, Kahn SE: Normal insulin secretion in humans. In: The International Textbook of Diabetes Mellitus, 3rd edition, RA DeFronzo, E Ferrannini, H Keen, and P Zimmet, eds., John Wiley and Sons, Chichester England, 2004, p 139-151.

19. Utzschneider KM, Kahn SE: ß-cell dysfunction in type 2 diabetes. In: The International Textbook of Diabetes Mellitus, 3rd edition, RA DeFronzo, E Ferrannini, H Keen, and P Zimmet, eds., John Wiley and Sons, Chichester England, 2004, p 375-385.

20. Chait A, Lewis K, Tannock L, O'Brien K, Retzlaff B, Kahn S, Knopp R, Wight T: Nutrition and inflammation: role of dietary cholesterol. In: Atherosclerosis XIII. Proceedings of the 13th International Atherosclerosis Symposium, Kyoto, Japan, Y Matsuzawa, T Kita, R Nagai, T Teramoto, eds., Elsevier, Amsterdam, ICS#1262, 2004, p 313-316.

21. Røder ME, Kahn SE: Suppression of ß-cell secretion by somatostatin does not fully reverse the disproportionate proinsulinemia of type 2 diabetes. Diabetes 53 (Suppl 3): S22-S25; 2004.

22. Kahn SE, Porte D Jr: The pathophysiology and genetics of type 2 diabetes mellitus. In: The Diabetes Mellitus Manual, SE Inzucchi, D Porte Jr, RS Sherwin and AD Baron, eds., McGraw-Hill, New York, 2004, p 51-75.

23. Porte D Jr, Kahn SE: New and old thoughts on hyperproinsulinemia and amyloid in type 2 diabetes. In: Commentaries on Perspectives in Diabetes – Volume 1 (1988-1992), RP Robertson, ed., American Diabetes Association, Alexandria, VA, 2006, p 70-71.

24. Kahn SE, Andrikopoulos S, Hull RL, Verchere CB: Islet amyloid: a long recognized but now more appreciated pathological feature of type 2 diabetes mellitus. In: Commentaries on Perspectives in Diabetes – Volume 3 (1998-2002), RP Robertson, ed., American Diabetes Association, Alexandria, VA, 2007, p 112-114.

25. Halban PA, Kahn SE, Lernmark A, Rhodes CJ: ß-cell replacement therapy: a roadmap with milestones but no shortcuts. In: Commentaries on Perspectives in Diabetes – Volume 3 (1998-2002), RP Robertson, ed., American Diabetes Association, Alexandria, VA, 2007, p 293-294.

26. Hayashi T, Boyko EJ, McNeely MJ, Leonetti DL, Kahn SE, Fujimoto WY: Response to Oda E re Minimum waist and visceral fat values for identifying Japanese Americans at risk for the metabolic syndrome (letter). Diabetes Care 30: e41; 2007 (DOI: 10.2337/dc07-0207).

27. Utzschneider KM, Kahn SE: Insulin action and ß-cell function: role in metabolic regulation. In: Metabolic Risk for Cardiovascular Disease, RH Eckel, ed., Wiley-Blackwell, Oxford, in press.

A Diabetes Outcome Progression Trial (ADOPT) Publications

1. Viberti G, Kahn SE, Greene DA, Herman WH, Zinman B, Holman RR, Haffner SM, Levy D, Lachin JM, Berry RA, Heise MA, Jones NP, Freed MI: A Diabetes Outcome Progression Trial (ADOPT). An international multicenter study of the comparative efficacy of rosiglitazone, glyburide, and metformin in recently diagnosed type 2 diabetes. Diabetes Care 25:1737-1743; 2002.

2. Zinman B, Kahn SE, Haffner SM, O'Neill MC, Heise MA, Freed MI, ADOPT Study Group: Phenotypic characteristics of GAD antibody-positive recently diagnosed patients with type 2 diabetes in North America and Europe. Diabetes 53: 3193-3200; 2004.

3. Kahn SE, Zinman B, Haffner SM, O'Neill MC, Kravitz BG, Yu D, Freed MI, Herman WH, Holman RR, Jones NP, Lachin JM, Viberti GC, ADOPT Study Group: Obesity is a major determinant of the association of C-reactive protein levels and the metabolic syndrome in type 2 diabetes. Diabetes 55:2357-2364; 2006.

4. Viberti G, Lachin JM, Holman RR, Zinman B, Haffner SM, Kravitz B, Heise MA, Jones NP, O'Neill MC, Freed MI, Kahn SE, Herman WH, ADOPT Study Group: A Diabetes Outcome Progression Trial (ADOPT): Baseline characteristics of type 2 diabetic patients in North America and Europe. Diabetic Medicine 23:1289-1294; 2006.

5. Kahn SE, Haffner SM, Heise MA, Herman WH, Holman RR, Jones NP, Kravitz BG, Lachin JM, O'Neill MC, Zinman B, Viberti G, ADOPT Study Group: Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. N Engl J Med 355:2427-2443; 2006. (Erratum 356:1387-1388; 2007).

6. Kahn SE, Viberti G, ADOPT Steering Committee: Glycemic durability of monotherapy for diabetes (letter). N Engl J Med 356:1380; 2007.

7. Kahn SE, Zinman B, Lachin JM, Haffner SM, Herman WH, Holman RR, Kravitz BG, Yu D, Heise MA, Aftring RP, Viberti G, ADOPT Study Group: Rosiglitazone-associated fractures in type 2 diabetes: an analysis from A Diabetes Outcome Progression Trial (ADOPT). Diabetes Care 31:845-851; 2008.

Diabetes Prevention Program (DPP) Publications

1. The Diabetes Prevention Program Research Group: The Diabetes Prevention Program: design and methods for a clinical trial in the prevention of type 2 diabetes. Diabetes Care 22:623-634; 1999.

2. The Diabetes Prevention Program Research Group: The Diabetes Prevention Program: evaluation and management of diabetes (letter). Diabetes Care 22:1757-1758; 1999.

3. Fujimoto WY, The Diabetes Prevention Program Research Group: Background and recruitment data for the U.S. Diabetes Prevention Program. Diabetes Care 23 (Suppl 2):B11-B13; 2000.

4. The Diabetes Prevention Program Research Group: The Diabetes Prevention Program: Baseline characteristics of the randomized cohort. Diabetes Care 23:1619-1629; 2000.

5. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM, The Diabetes Prevention Program Research Group: Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med 346:393-403; 2002.

6. Rubin RR, Fujimoto WY, Marrero DG, Brenneman T, Charleston JB, Edelstein SL, Fisher EB, Jordan R, Knowler WC, Lichterman LC, Prince M, Rowe PM, The Diabetes Prevention Program Research Group: The Diabetes Prevention Program: recruitment methods and results. Control Clin Trials 23:157-171; 2002.

7. Knowler WC, The Diabetes Prevention Program Research Group: Diabetes prevention (letter). N Engl J Med 346:1830; 2002.

8. Wylie-Rosett J, Delahanty L, The Diabetes Prevention Program Research Group: An integral role of the dietitian: implications of the Diabetes Prevention Program. J Am Diet Assoc 102:1065-1068; 2002

9. The Diabetes Prevention Program Research Group: Hypertension, insulin and proinsulin in participants with impaired glucose tolerance. Hypertension 40:679-686; 2002.

10. Delahanty LM, Meigs JB, Hatyden D, Williamson DA, Nathan DM, The Diabetes Prevention Program Research Group: Psychological and behavioral correlates of baseline BMI in the Diabetes Prevention Program. Diabetes Care 25:1992-1998; 2002.

11. The Diabetes Prevention Program Research Group*: The Diabetes Prevention Program: description of lifestyle intervention. Diabetes Care 25:2165-2171; 2002.

12. The Diabetes Prevention Program Research Group: Costs associated with the primary prevention of type 2 diabetes mellitus in the Diabetes Prevention Program. Diabetes Care 26:36-47; 2003.

13. The Diabetes Prevention Program Research Group*: Effects of withdrawal from metformin on the development of diabetes in the Diabetes Prevention Program. Diabetes Care 26:977-980; 2003.

14. The Diabetes Prevention Program Research Group: Within-trial cost-effectiveness of lifestyle intervention or metformin for the primary prevention of type 2 diabetes. Diabetes Care 26:2518-2523; 2003.

15. Molitch ME, The Diabetes Prevention Program Research Group*: Withdrawal from metformin in the Diabetes Prevention Program (letter). Diabetes Care 26:2702-2703; 2003.

16. Molitch ME, Fujimoto WY, Hamman RF, Knowler WC, The Diabetes Prevention Program Research Group: The Diabetes Prevention Program and its global implications. J Am Soc Nephrol 14 (Suppl 2):S103-S107; 2003.

17. Nathan DM, Hamman RF, Garfield S, Brenneman A, Fowler SF, Knowler WC, The Diabetes Prevention Program Research Group: Preventing Type 2 Diabetes: The Diabetes Prevention Program. In: Diabetes Mellitus: A Fundamental and Clinical Text, 3rd edition, D LeRoith, SI Taylor, and JM Olefsky, eds., Lippincott, Williams and Wilkins, Philadelphia, 2004, p 1077-1084.

18. The Diabetes Prevention Program Research Group: Achieving weight and activity goals amongst Diabetes Prevention Program lifestyle participants. Obes Res 12:1426-1434; 2004.

19. Mayer-Davis EJ, Sparks KC, Hirst K, Costacou T, Lovejoy JC, Regensteiner JG, Hoskin MA, Kriska AM, Bray GA, The Diabetes Prevention Program Research Group: Dietary intake in the Diabetes Prevention Program cohort: baseline and 1-year post-randomization. Ann Epidemiol 14: 763-772; 2004.

20. The Diabetes Prevention Program Research Group: Strategies to identify adults at high risk for type 2 diabetes: The Diabetes Prevention Program. Diabetes Care 28:138-144; 2005.

21. Herman WH, Hoerger TJ, Brandle M, Hicks K, Sorensen S, Zhang P, Hamman RF, Ackermann RT, Engelgau MM, Ratner RE, The Diabetes Prevention Program Research Group: The cost-effectiveness of lifestyle modification or metformin in preventing type 2 diabetes in adults with impaired glucose tolerance. Ann Intern Med 142:323-332; 2005.

22. The Diabetes Prevention Program Research Group*: Prevention of type 2 diabetes with troglitazone in the Diabetes Prevention Program. Diabetes 54:1150–1156, 2005.

23. The Diabetes Prevention Program Research Group: Depression symptoms and antidepressant medication use in the Diabetes Prevention Program participants. Diabetes Care 28:830–837, 2005.

24. The Diabetes Prevention Program Research Group: Impact of intensive lifestyle and metformin therapy on cardiovascular risk factors in the Diabetes Prevention Program. Diabetes Care 28:888–894, 2005.

25. Orchard TJ, Temprosa M, Goldberg R, Haffner S, Ratner R, Marcovina S, Fowler S, The Diabetes Prevention Program Research Group: The effect of metformin and intensive lifestyle intervention on the metabolic syndrome: the Diabetes Prevention Program randomized trial. Ann Intern Med 142:611-619; 2005.

26. The Diabetes Prevention Research Group: Intensive lifestyle intervention or metformin on inflammation and coagulation in participants with impaired glucose tolerance. Diabetes 54:1566-1572; 2005.

27. The Diabetes Prevention Research Group*: Role of insulin secretion and sensitivity in the evolution of type 2 diabetes in the Diabetes Prevention Program: Effects of lifestyle intervention and metformin. Diabetes 54:2404-2414; 2005.

28. The Diabetes Prevention Program Research Group: Lipid, lipoproteins, C-reactive protein, and hemostatic factors at baseline in the Diabetes Prevention Program. Diabetes Care 28:2472–2479, 2005.

29. Brown JS, Wing R, Barrett-Connor E, Nyberg LM, Kusek JW, Orchard TJ, Ma Y, Vittinghoff E, Kanaya AM, The Diabetes Prevention Program Research Group: Lifestyle intervention is associated with lower prevalence of urinary incontinence: The Diabetes Prevention Program. Diabetes Care 29:385-390; 2006.

30. Ratner RE, The Diabetes Prevention Program Research Group: An update on the Diabetes Prevention Program. Endocr Pract 12 (Suppl 1):20-24; 2006.

31. Carnethon MR, Prineas RJ, Temprosa M, Zhang ZM, Uwaifo G, Molitch ME, The Diabetes Prevention Program Research Group: The association among autonomic nervous system function, incident diabetes, and intervention arm in the Diabetes Prevention Program. Diabetes Care 29:914-919; 2006.

32. Kriska AM, Edelstein SL, Hamman RF, Otto A, Bray GA, Mayer-Davis EJ, Wing RR, Horton ES, Haffner SM, Regensteiner JG, The Diabetes Prevention Program Research Group: Physical activity in individuals at risk for diabetes: Diabetes Prevention Program. Med Sci Sports Exerc 38:826-832; 2006.

33. Delahanty LM, Conroy MB, Nathan DM, The Diabetes Prevention Program Research Group: Psychological predictors of physical activity in the Diabetes Prevention Program. J Am Diet Assoc 106:698-705; 2006.

34. Florez JC, Jablonski KA, Bayley N, Pollin TI, de Bakker PIW, Shuldiner AR, Knowler WC, Nathan DM, Altshuler D, The Diabetes Prevention Program Research Group: *TCF7L2* polymorphisms and progression to diabetes in the Diabetes Prevention Program. N Engl J Med 355:241-250; 2006.

35. Walker EA, Molitch M, Kramer MK, Kahn SE, Ma Y, Edelstein S, Smith K, Johnson MK, Kitabchi A, Crandall J, The Diabetes Prevention Program Research Group: Adherence to preventive medications: predictors and outcomes in the Diabetes Prevention Program. Diabetes Care 29:1997-2002; 2006

36. Hamman RF, Wing RR, Edelstein SL, Lachin JM, Bray GA, Delahanty L, Hoskin M, Kriska AM, Mayer-Davis EJ, Pi-Sunyer X, Regensteiner J, Venditti B, Wylie-Rosett J, The Diabetes Prevention Program Research Group: Effect of weight loss with lifestyle intervention on risk of diabetes. Diabetes Care 29:2102-2107; 2006.

37. The Diabetes Prevention Program Research Group: The influence of age on the effects of lifestyle modification and metformin in prevention of diabetes. J Gerontol A Biol Sci Med 61:1075-1081; 2006.

38. The Diabetes Prevention Program Research Group: Relationship of body size and shape to the development of diabetes in the Diabetes Prevention Program. Obesity 14:2107-2117; 2006.

39. The Diabetes Prevention Research Group: The prevalence of retinopathy in impaired glucose tolerance and recent-onset diabetes in the Diabetes Prevention Program. Diabet Med 24:137-144; 2007.

40. Florez JC, Jablonski KA, Kahn SE, Franks PW, Dabelea D, Hamman RF, Knowler WC, Nathan DM, Altshuler D, The Diabetes Prevention Program Research Group: Type 2 diabetes-associated missense polymorphisms *KCNJ11* E23K and *ABCC8* A1369S influence progression to diabetes and response to interventions in the Diabetes Prevention Program. Diabetes 56:531-536; 2007.

41. Lachin JM, Christophi CA, Edelstein SL, Ehrmann DA, Hamman RF, Kahn SE, Knowler WC, Nathan DM, The Diabetes Prevention Research Group: Factors associated with diabetes onset during metformin versus placebo therapy in the Diabetes Prevention Program. Diabetes 56:1153-1159; 2007.

42. Florez JC, Jablonski K, Sun MW, Bayley N, Kahn SE, Shamoon H, Hamman RF, Knowler WC, Nathan DM, Altshuler D, The Diabetes Prevention Program Research Group: Effects of the type 2 diabetes-associated *PPARG* P12A polymorphism on progression to diabetes and response to troglitazone. J Clin Endocrinol Metab 92:1502-1509; 2007.

43. Fujimoto WY, Jablonski KA, Bray GA, Kriska A, Barrett-Connor E, Haffner S, Hanson R, Hill JO, Hubbard V, Stamm E, Pi-Sunyer X, The Diabetes Prevention Program Research Group: Body size and shape changes and the risk of diabetes in the Diabetes Prevention Program. Diabetes 56:1680-1685; 2007.

44. Herman WH, Ma Y, Uwaifo G, Haffner S, Kahn SE, Horton ES, Lachin JM, Montez MG, Brenneman T, Barrett-Connor E, The Diabetes Prevention Program Research Group: Differences in A1c by race and ethnicity among patients with impaired glucose tolerance in the Diabetes Prevention Program. Diabetes Care 30:2453-2457; 2007.

45. Nathan DM, The Diabetes Prevention Program Research Group: Diabetic retinopathy without diabetes (letter). Diabet Med 24:1174-11175; 2007.

46. Franks PW, Jablonski KA, Delahanty L, Hanson RL, Kahn SE, Altshuler D, Knowler WC, Florez JC, The Diabetes Prevention Program Research Group: The Pro12Ala variant at the peroxisome proliferator-activated receptor γ gene and change in obesity-related traits in the Diabetes Prevention Program. Diabetologia 50:2451-2460; 2007.

47. Rubin RR, Ma Y, Marrero DG, Peyrot M. Barrett-Connor EL, Kahn SE, Haffner SM, Price DW, Knowler WC, The Diabetes Prevention Program Research Group: Elevated depression symptoms, antidepressant medicine use and risk of developing diabetes during the Diabetes Prevention Program. Diabetes Care 31:420-426; 2008.

48. Florez JC, Jablonski KA, McAteer J, Sandhu MS, Wareham NJ, Franks PW, Altshuler D, Knowler WC, The Diabetes Prevention Program Research Group: Testing of diabetes-associated WFS1 polymorphisms in the Diabetes Prevention Program. Diabetologia 51:451-457; 2008.

49. Mather KJ, Funahashi T, Matsuzawa Y, Edelstein S, Bray GA, Kahn SE, Crandall J, Marcovina S, Goldstein B, Goldberg R, The Diabetes Prevention Program Research Group: Adiponectin, change in adiponectin, and progression to diabetes in the Diabetes Prevention Program. Diabetes 57:980-986; 2008.

50. Bray GA, Jablonski KA, Fujimoto WY, Barrett-Connor E, Haffner S, Hanson RL, Hill JO, Hubbard V, Kriska A, Stamm E, Pi-Sunyer FX, The Diabetes Prevention Program Research Group: Relation of central adiposity and body mass index to the development of diabetes in the Diabetes Prevention Program. Am J Clin Nutr 87:1212-1218; 2008.

51. Crandall JP, Knowler WC, Kahn SE, Marrero D, Florez JC, Bray GA, Haffner SM, Hoskin M, Nathan DM, The Diabetes Prevention Program Research Group: The prevention of type 2 diabetes. Nat Clin Pract Endocrinol Metab 4:382-393; 2008.

52. Perreault L, Ma Y, Dagogo-Jack S, Horton E, Marrero D, Crandall J, Barrett-Connor E, The Diabetes Prevention Program Research Group: Sex differences in diabetes risk and the effect of intensive lifestyle modification in the Diabetes Prevention Program. Diabetes Care 31:1416-1421; 2008.

53. Moore AF, Jablonski KA, McAteer JB, Pollin TI, Franks PW, Hanson RL, Shuldiner AR, Knowler WC, Altshuler D, Florez JC, The Diabetes Prevention Program Research Group: Extension of type 2 diabetes genome-wide association scan results in the Diabetes Prevention Program. Diabetes 57:2503-2510; 2008.

54. Franks PW, Jablonski KA, Delahanty LM, McAteer JB, Kahn SE, Knowler WC, Florez JC, The Diabetes Prevention Program Research Group: Assessing gene-treatment interactions at the *FTO* and *INSIG2* loci on obesity-related traits in the Diabetes Prevention Program. Diabetologia 51:2214-2223; 2008.

55. Friedman AN, Marrero D, Ma Y, Ackermann R, Narayan V, Barrett-Connor E, Watson K, Knowler WC, Horton ES, The Diabetes Prevention Program Research Group: Value of urinary albumin-to-creatinine ratio as a predictor of type 2 diabetes in pre-diabetic individuals. Diabetes Care 31:2344-2348; 2008.

56. Ratner RE, Christophi CA, Metzger BE, Dabelea D, Bennett PH, Pi-Sunyer X, Fowler S, Kahn SE, The Diabetes Prevention Program Research Group: Prevention of diabetes in women with a history of gestational diabetes: effects of metformin and lifestyle interventions. J Clin Endocrinol Metab 93:4774-4779; 2008.

57. Moore AF, Jablonski KA, Mason CC, McAteer JB, Arakaki RF, Goldstein BJ, Kahn SE, Kitabchi WC, Hanson RL, Knowler WC, Florez JC, The Diabetes Prevention Program Research Group: The association of *ENPP1* K121Q with diabetes incidence is abolished by lifestyle modification in the Diabetes Prevention Program. J Clin Endocrinol Metab, in press.

* Denotes member of the writing group

Genetics of NIDDM (GENNID) Study Group Publications

1. Raffel LJ, Robbins DC, Norris JM, Boerwinkle E, DeFronzo RA, Elbein SC, Fujimoto W, Hanis CL, Kahn SE, Permutt MA, Chiu KC, Cruz J, Ehrmann DA, Robertson RP, Rotter JI, Buse J: The GENNID Study: a resource for mapping the genes that cause NIDDM. Diabetes Care 19:864-872; 1996.

2. Ehm MG, Karnoub MC, Sakul H, Gottschalk K, Holt DC, Weber JL, Vaske D, Briley D, Briley L, Kopf J, McMillen P, Nguyen Q, Reisman M, Lai EH, Joslyn G, Shepherd NS, Bell C, Wagner MJ, Burns DK, American Diabetes Association GENNID Study Group: Genomewide search for type 2 diabetes susceptibility genes in four American populations. Am J Hum Genet 66:1871-1881; 2000.

3. Jensen CC, Cnop M, Hull RL, Fujimoto WY, Kahn SE, American Diabetes Association GENNID Study Group: ß-cell function is a major contributor to oral glucose tolerance in high-risk relatives of four ethnic groups in the U.S. Diabetes 51:2170-2178; 2002.

4. Duggirala R, Almasy L, Blangero J, Jenkinson CP, Arya R, DeFronzo RA, Stern MP, O'Connell P, American Diabetes Association GENNID Study Group: Further evidence for a type 2 diabetes susceptibility locus on chromosome 11q. Genetic Epidemiology 24:240-242; 2003.

5. Malhotra A, Wolford JK, American Diabetes Association GENNID Study Group: Analysis of quantitative lipid traits in the Genetics of NIDDM (GENNID) Study. Diabetes 54:3007-3014; 2005.

6. Malhotra A, Coon H, Feitosa MF, Li WD, North KE, Price RA, Bouchard C, Hunt SC, Wolford JK, American Diabetes Association GENNID Study Group: Meta-analysis of genome-wide linkage studies for quantitative lipid traits in African Americans. Hum Mol Genet 14:3955-3962; 2005.

7. Carr DB, Utzschneider KM, Hull RL, Tong J, Wallace TM, Kodama K, Shofer JB, Heckbert SR, Boyko EJ, Fujimoto WY, Kahn SE, American Diabetes Association GENNID Study Group: Gestational diabetes increases the risk of cardiovascular disease in women with a family history of type 2 diabetes. Diabetes Care 29:2078-2083; 2006.

8. van Genugten RE, Utzschneider KM, Tong J, Gerchman F, Zraika S, Udayasankar J, Boyko EJ, Fujimoto WY, Kahn SE, American Diabetes Association GENNID Study Group: Effects of sex and hormone replacement therapy use on the prevalence of isolated impaired fasting glucose and isolated impaired glucose tolerance in subjects with a family history of type 2 diabetes. Diabetes 55:3529-3535; 2006.

Look Ahead Publications

1. The Look AHEAD Research Group*: Look AHEAD (Action for Health in Diabetes): design and methods for a clinical trial of weight loss for the prevention of cardiovascular disease in type 2 diabetes. Control Clin Trials 24:610-628, 2003.

2. The Look AHEAD Research Group: The Look AHEAD study: a description of the lifestyle intervention and the evidence supporting it. Obesity 14:737-752; 2006.

3. Espeland MA, Dotson K, Jaramillo SA, Kahn SE, Harrison B, Montez M, Foreyt JP, Montgomery B, Knowler WC, The Look AHEAD Research Group: Consent for genetics studies among clinical trial participants: findings from Action for Health in Diabetes (Look AHEAD). Clin Trials 3:443-456; 2006.

4. Rejeski WJ, Lang W, Neiberg RH, Van Dorsten B, Foster GD, Maciejewski ML, Rubin R, Williamson DF, The Look AHEAD Research Group: Correlates of health-related quality of life in overweight and obese adults with type 2 diabetes. Obesity 14:870-883; 2006.

5. The Look AHEAD Research Group: Baseline characteristics of the randomised cohort from the Look AHEAD (Action for Health in Diabetes) study. Diab Vasc Dis Res 3:202-215; 2006.

6. The Look AHEAD Research Group: Reduction in weight and cardiovascular disease risk factors in individuals with type 2 diabetes: one-year results of the Look AHEAD trial. Diabetes Care 30:1374-1383; 2007.

7. Ribisl PM, Lang W, Jaramillo SA, Jakicic JM, Stewart KJ, Bahnson J, Bright R, Curtis JF, Crow RS, Soberman JE, The Look AHEAD Research Group: Exercise capacity and cardiovascular/metabolic characteristics of overweight and obese individuals with type 2 diabetes: The Look AHEAD clinical trial. Diabetes Care 30:2679-2684; 2007.

8. Wing RR, Jakicic J, Neiberg R, Lang W, Blair SN, Cooper L, Hill JO, Johnson KC, Lewis CE, The Look AHEAD Research Group: Fitness, fatness, and cardiovascular risk factors in type 2 diabetes: The Look AHEAD Study. Med Sci Sports Exerc 39:2107-2116; 2007.

9. Bertoni AG, Clark JM, Feeney P, Yanovski SZ, Bantle J, Montgomery B, Safford MM, Herman WH, Haffner S, The Look AHEAD Research Group: Suboptimal control of glycemia, blood pressure, and LDL cholesterol in overweight adults with diabetes: The Look AHEAD Study. J Diabetes Complications 22:1-9; 2008.

10. Heshka S, Ruggiero A, Bray GA, Foreyt J, Kahn SE, Lewis CE, Saad M, Schwartz AV, The Look AHEAD Research Group: Altered body composition in type 2 diabetes mellitus. Int J Obes, 32:780-787; 2008.

11. Raynor HA, Jeffery RW, Ruggiero AM, Clark JM, Delahanty LM, The Look AHEAD Research Group: Weight loss strategies associated with BMI in overweight adults with type 2 diabetes at entry into the Look AHEAD (Action for Health in Diabetes) Trial. Diabetes Care 31:1299-1304; 2008.

12. Espeland MA, Regensteiner JG, Jaramillo SA, Gregg E, Knowler WC, Wagenknecht LE, Bahnson J, Haffner S, Hill J, Hiatt WR, The Look AHEAD Research Group. Measurement characteristics of the ankle-brachial index: results from the Action for Health in Diabetes study. Vasc Med 13:225-233; 2008.

* Denotes member of the writing group

Nateglinide and Valsartan in Impaired Glucose Tolerance Outcomes Research (NAVIGATOR) Publications

1. Califf RM, Boolell M, Haffner SM, Bethel MA, McMurray J, Duggal A, Holman RR, NAVIGATOR Study Group: Prevention of diabetes and cardiovascular disease in patients with impaired glucose tolerance: Rationale and design of the Nateglinide and Valsartan in Impaired Glucose Tolerance Outcomes Research (NAVIGATOR) Trial. Am Heart J 156:623-632; 2008.

APPENDIX 2

US006294170B1

(12) **United States Patent**
Boone et al.

(10) **Patent No.: US 6,294,170 B1**
(45) **Date of Patent: Sep. 25, 2001**

(54) **COMPOSITION AND METHOD FOR TREATING INFLAMMATORY DISEASES**

(75) Inventors: **Thomas C. Boone; Susan Hershenson**, both of Newbury Park, CA (US); **Michael P. Bevilacqua**, Boulder, CO (US); **David S. Collins**, Fishers, IN (US)

(73) Assignee: **Amgen Inc.**, Thousand Oaks, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/131,247**

(22) Filed: **Aug. 7, 1998**

Related U.S. Application Data

(60) Provisional application No. 60/055,185, filed on Aug. 8, 1997.

(51) **Int. Cl.**[7] **A61K 38/00**; A61K 39/395
(52) **U.S. Cl.** **424/134.1**; 514/12; 530/324
(58) **Field of Search** 424/134.1; 514/12; 530/324

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,843,778	*	12/1998	Rosenberg et al.	435/235
5,858,355		1/1999	Glorioso et al.	424/93.21

FOREIGN PATENT DOCUMENTS

9173636	10/1991	(AU) .
WO 92/11359	7/1992	(WO) .
WO 93/07863	4/1993	(WO) .

OTHER PUBLICATIONS

Chang et al. Pharmaceutical Research, 13:243–249, Feb. 1996.*
Kato et al. Vet. Immunol. Immunopathol. (1997), 56(3–4), 221–231, Mar. 1997.*
Chang et al. Immunol. Invest. (1996), 25(4), 355–368, Apr. 1996.*
Hsu et al.Transplant. Proc. (1996), 28(3), 1961–1963, Mar. 1996.*
Cominelli et al. J. Biol. Chem. (1994), 269(9), 6962–71, Sep. 1994.*
Shuck et al. Eur. J. Immunol. (1991), 21(11), 2775–80, Nov. 1991.*

* cited by examiner

Primary Examiner—Michael Born
(74) *Attorney, Agent, or Firm*—Timothy J. Gaul; Ron K. Levy; Steven M. Odre

(57) **ABSTRACT**

A protein which exhibits a therapeutic effect on inflammation and is useful for treating IL-1-mediated inflammatory diseases, particularly diseases of the joint.

15 Claims, 14 Drawing Sheets

FIG. 1

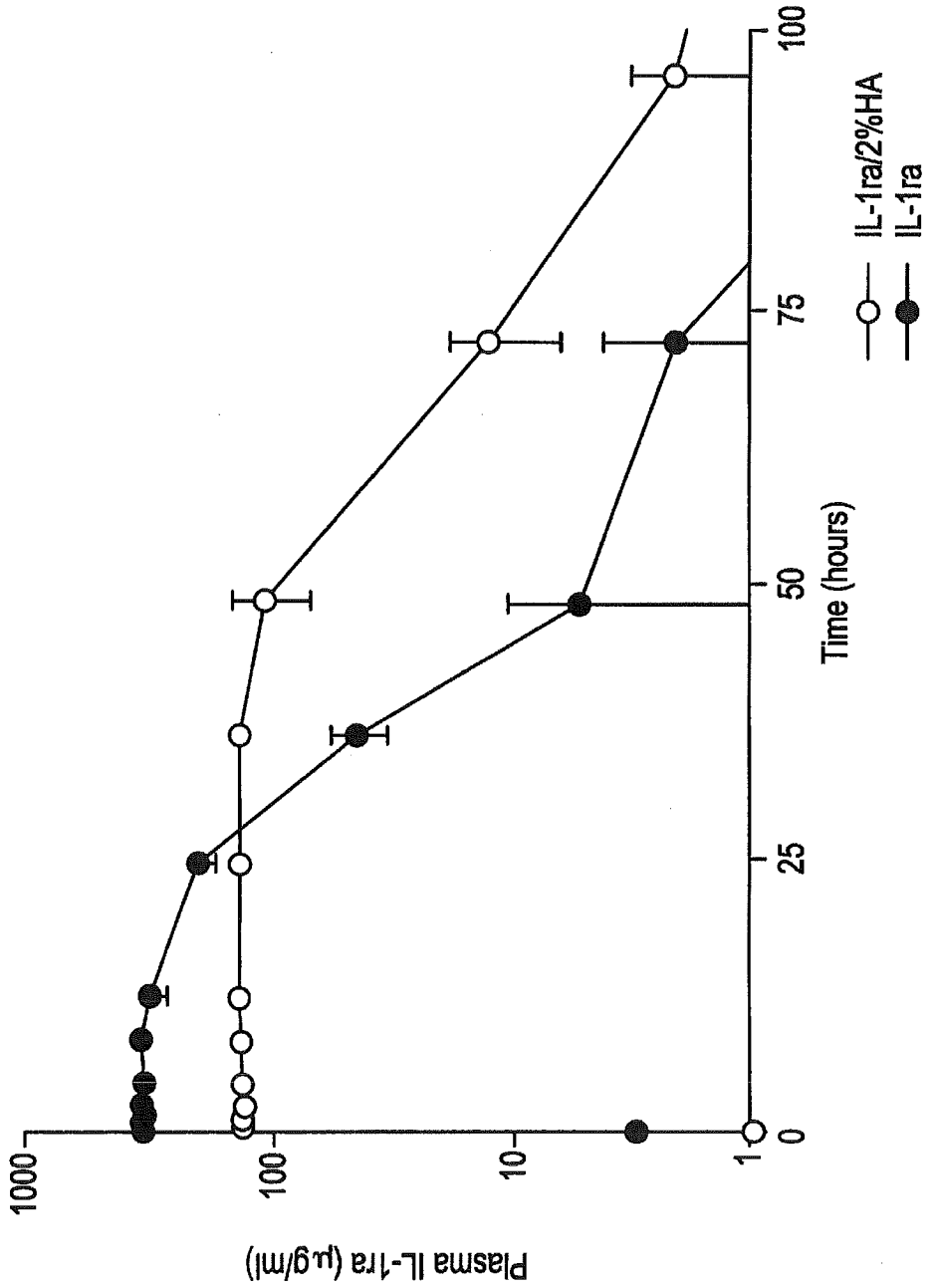

Plasma IL-1ra (μg/ml)
1000
100
10
1
0
25
50
75
100
Time (hours)
IL-1ra/2%HA
IL-1ra

FIG. 2

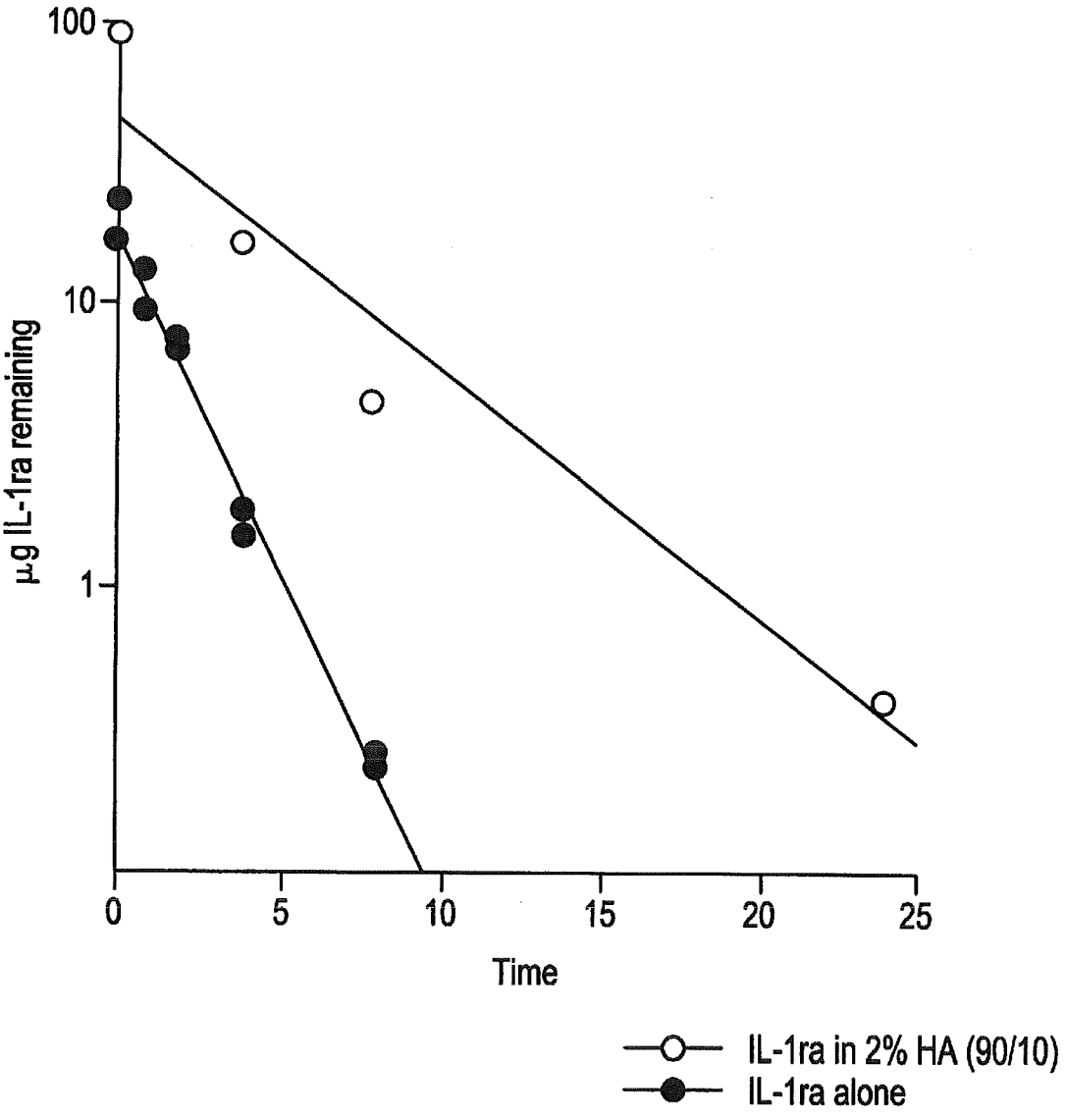

100
10
1
μg IL-1ra remaining
0
5
10
15
20
25
Time
IL-1ra in 2% HA (90/10)
IL-1ra alone

FIG. 3

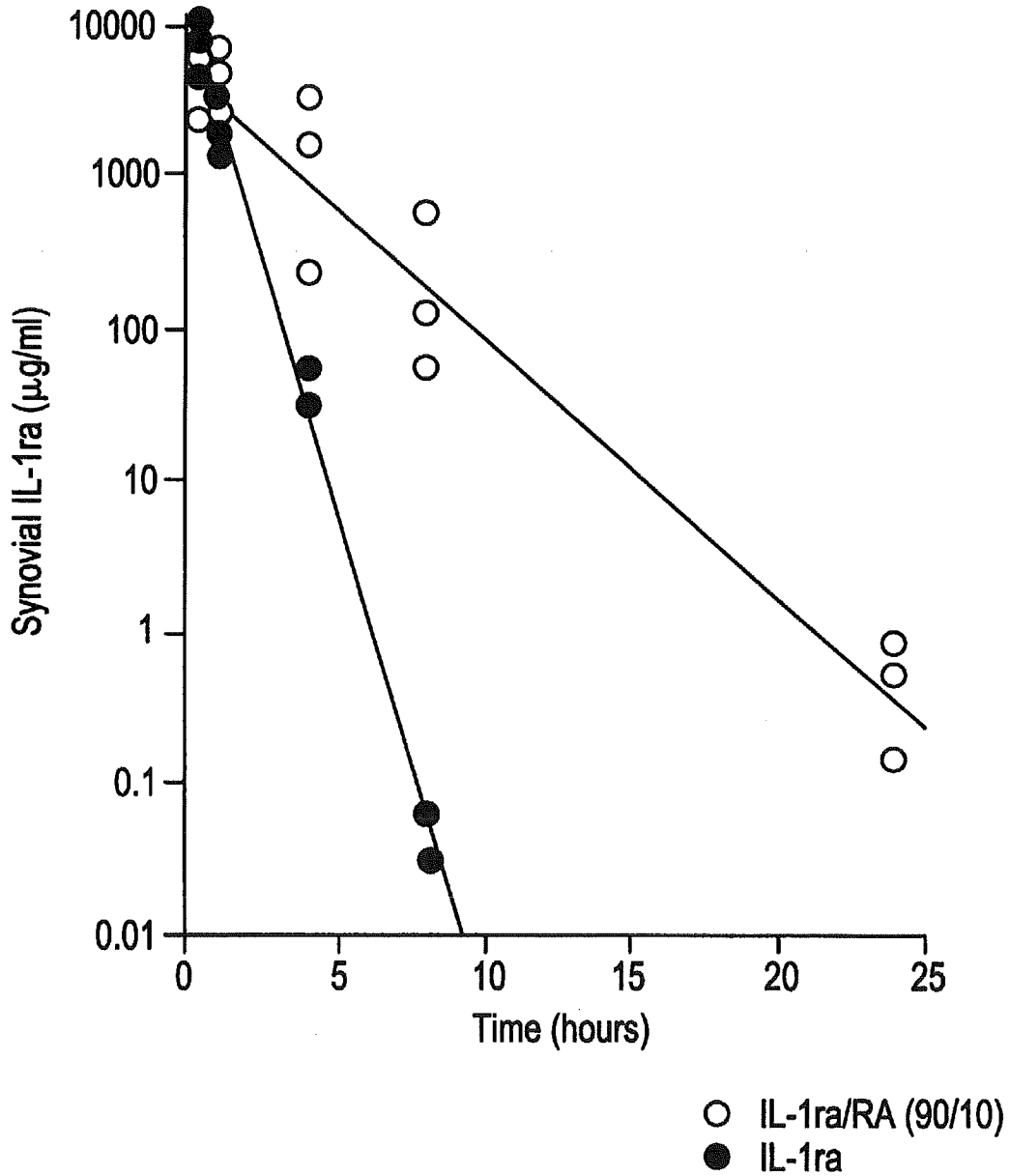

10000
1000
100
10
1
0.1
0.01
Synovial IL-1ra (µg/ml)
0
5
10
15
20
25
Time (hours)
IL-1ra/RA (90/10)
IL-1ra

FIG. 4

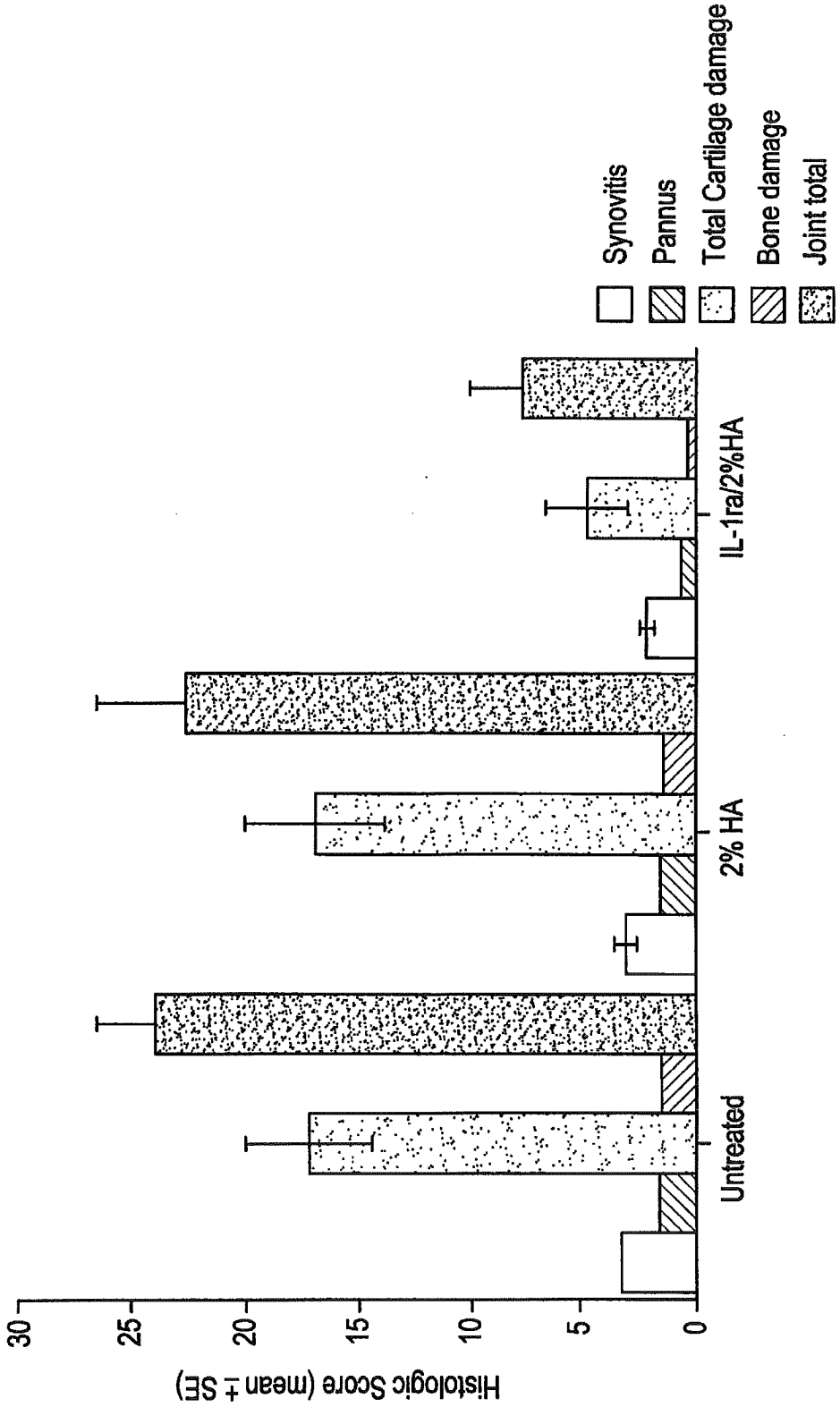

Synovitis
Pannus
Total Cartilage damage
Bone damage
Joint total
Histologic Score (mean ± SE)
30
25
20
15
10
5
0
Untreated
2% HA
IL-1ra/2%HA

FIG. 5

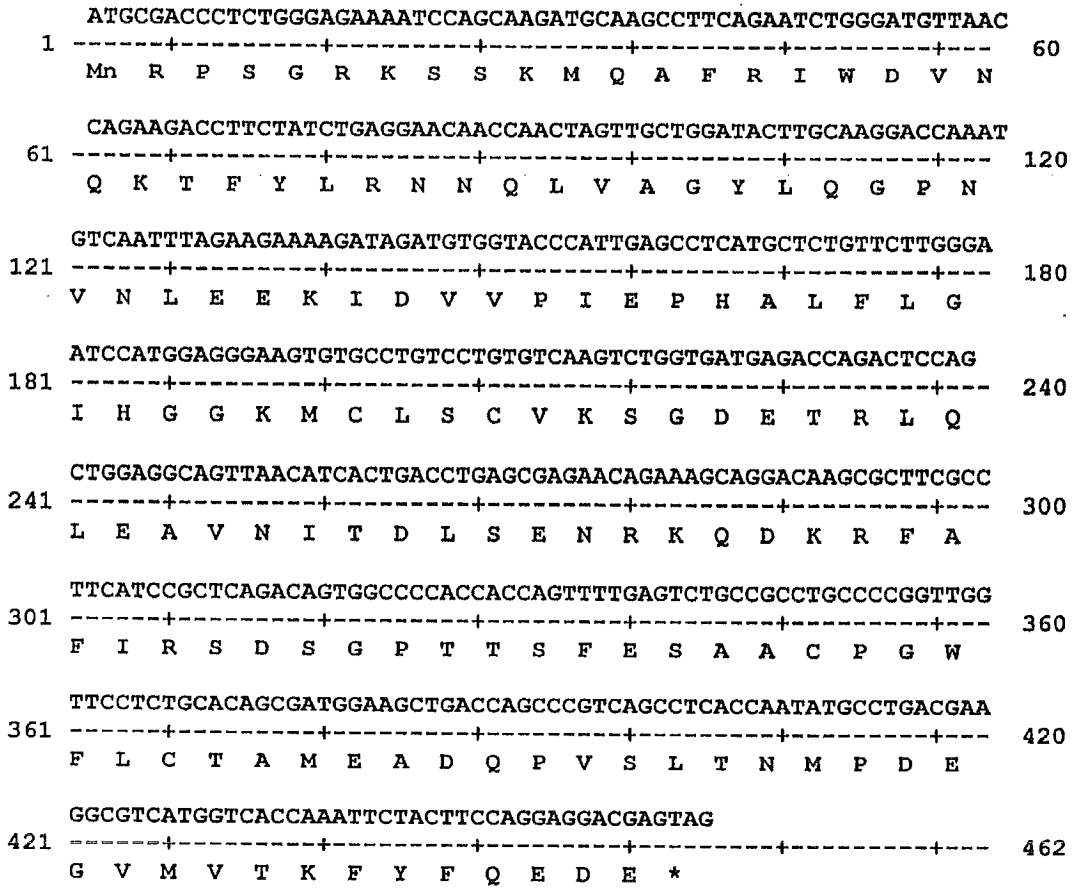

ATGCGACCCTCTGGGAGAAAATCCAGCAAGATGCAAGCCTTCAGAATCTGGGATGTTAAC
1
60
Mn R P S G R K S S K M Q A F R I W D V N
CAGAAGACCTTCTATCTGAGGAACAACCAACTAGTTGCTGGATACTTGCAAGGACCAAAT
61
120
Q K T F Y L R N N Q L V A G Y L Q G P N
GTCAATTTAGAAGAAAAGATAGATGTGGTACCCATTGAGCCTCATGCTCTGTTCTTGGGA
121
180
V N L E E K I D V V P I E P H A L F L G
ATCCATGGAGGGAAGTGTGCCTGTCCTGTGTCAAGTCTGGTGATGAGACCAGACTCCAG
181
240
I H G G K M C L S C V K S G D E T R L Q
CTGGAGGCAGTTAACATCACTGACCTGAGCGAGAACAGAAAGCAGGACAAGCGCTTCGCC
241
300
L E A V N I T D L S E N R K Q D K R F A
TTCATCCGCTCAGACAGTGGCCCCACCACCAGTTTTGAGTCTGCCGCCTGCCCCGGTTGG
301
360
F I R S D S G P T T S F E S A A C P G W
TTCCTCTGCACAGCGATGGAAGCTGACCAGCCCGTCAGCCTCACCAATATGCCTGACGAA
361
420
F L C T A M E A D Q P V S L T N M P D E
GGCGTCATGGTCACCAAATTCTACTTCCAGGAGGACGAGTAG
421
462
G V M V T K F Y F Q E D E *

FIG. 6

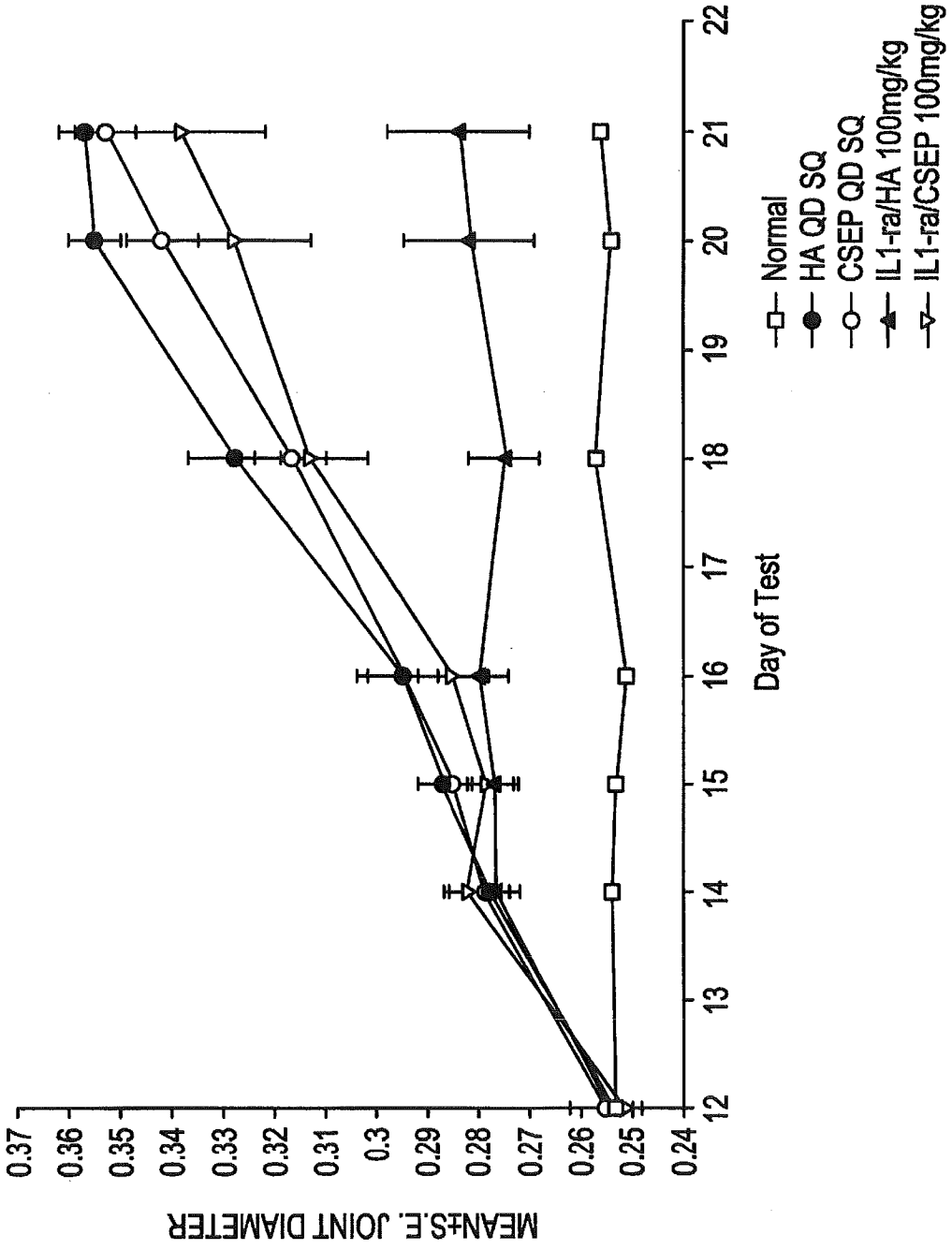

MEAN±S.E. JOINT DIAMETER
0.37
0.36
0.35
0.34
0.33
0.32
0.31
0.3
0.29
0.28
0.27
0.26
0.25
0.24
12
13
14
15
16
17
18
19
20
21
22
Day of Test
Normal
HA QD SQ
CSEP QD SQ
IL1-ra/HA 100mg/kg
IL1-ra/CSEP 100mg/kg

FIG. 7

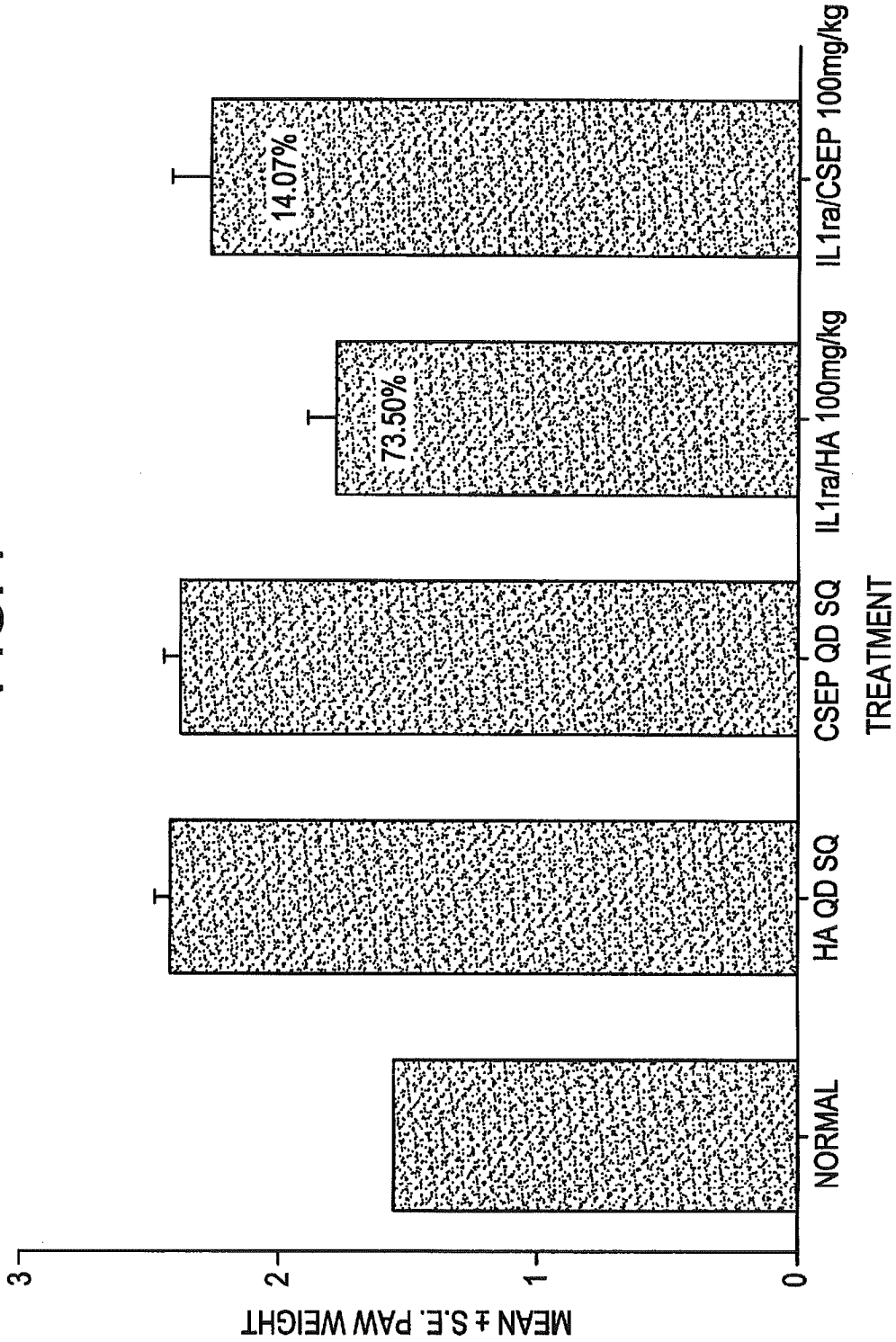

MEAN ± S.E. PAW WEIGHT
0
1
2
3
NORMAL
HA QD SQ
CSEP QD SQ
IL1ra/HA 100mg/kg
IL1ra/CSEP 100mg/kg
TREATMENT
-73.50%
-14.07%

FIG. 8

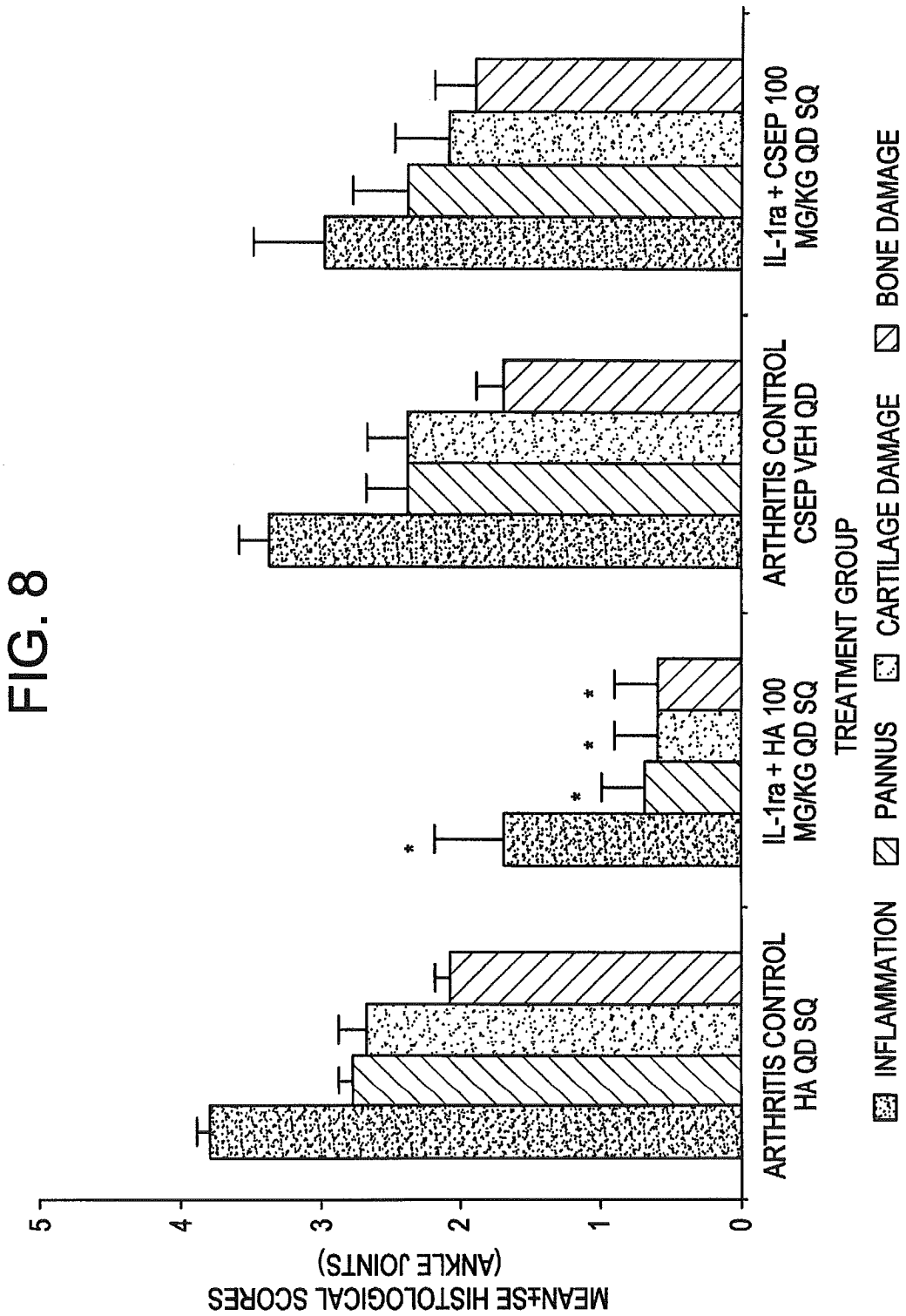

MEAN±SE HISTOLOGICAL SCORES
(ANKLE JOINTS)
0
1
2
3
4
5
*
*
*
ARTHRITIS CONTROL HA QD SQ
IL-1ra + HA 100 MG/KG QD SQ
ARTHRITIS CONTROL CSEP VEH QD
IL-1ra + CSEP 100 MG/KG QD SQ
TREATMENT GROUP
INFLAMMATION
PANNUS
CARTILAGE DAMAGE
BONE DAMAGE

FIG. 9

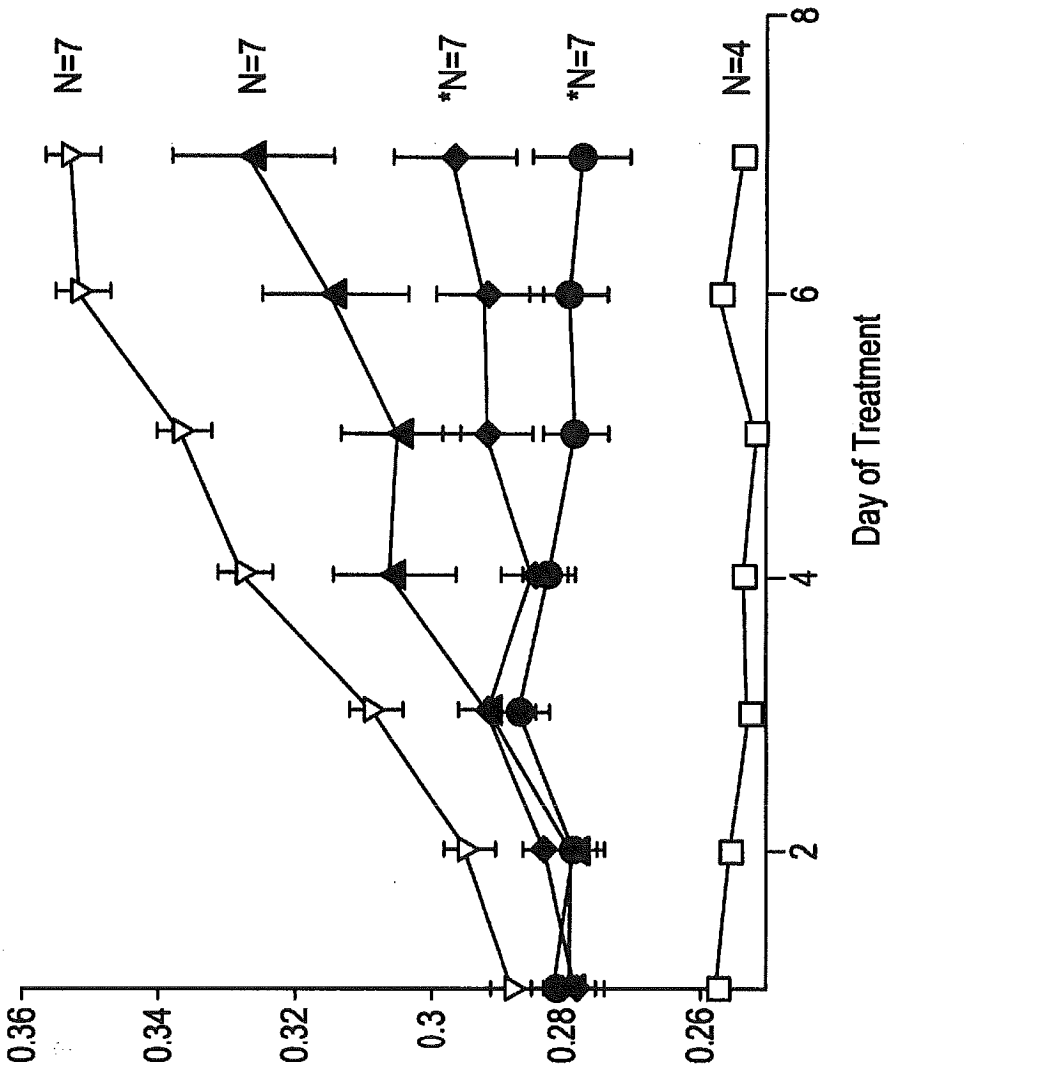

Disease control
IL-1ra/HA q72hr
IL-1ra/HA q48hr
IL-1ra/HA q24hr
Normal
N=7
N=7
*N=7
*N=7
N=4
0.36
0.34
0.32
0.3
0.28
0.26
2
4
6
8
Day of Treatment

FIG. 10

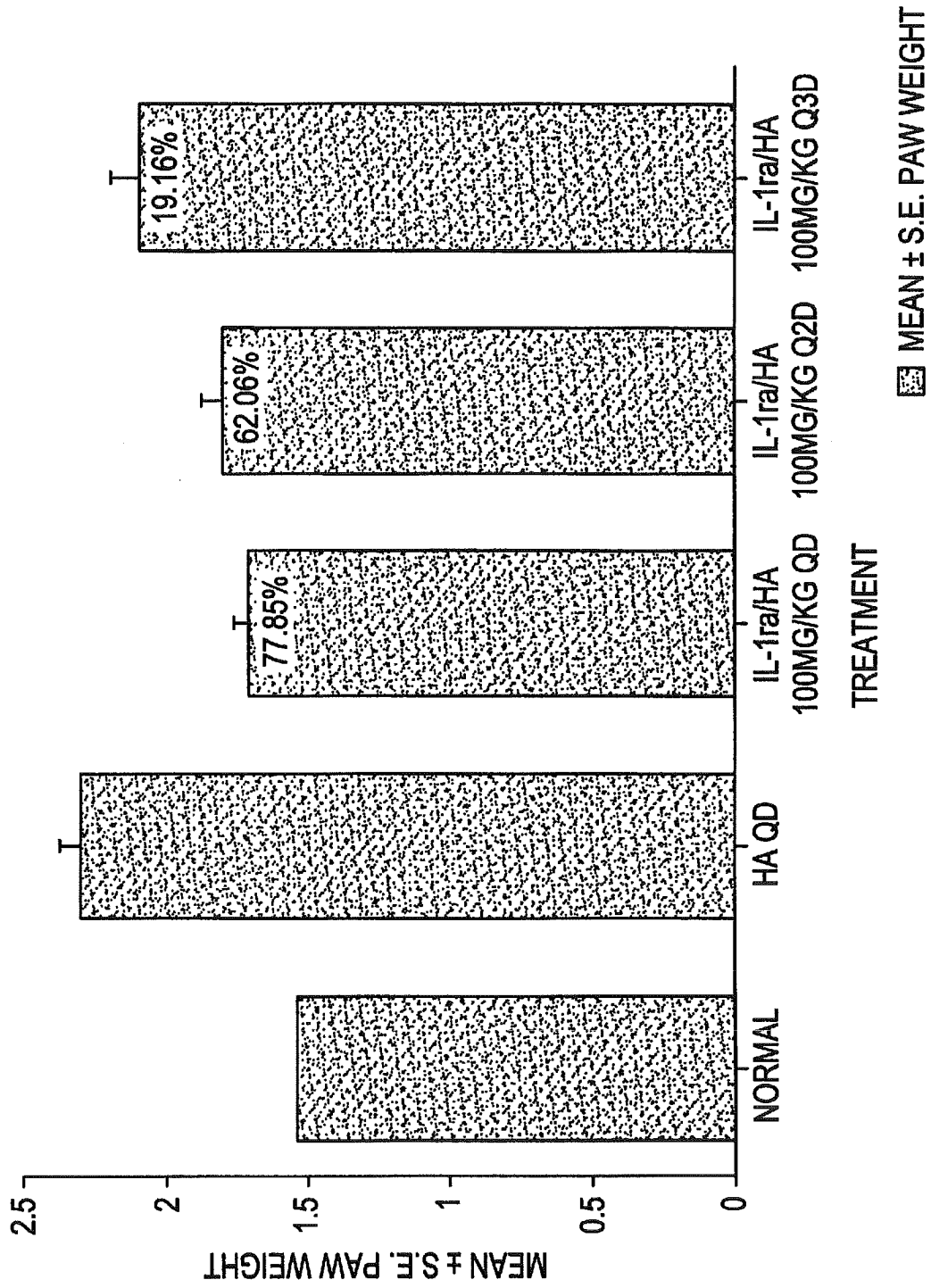

MEAN ± S.E. PAW WEIGHT
2.5
2
1.5
1
0.5
0
NORMAL
HA QD
IL-1ra/HA 100MG/KG QD
IL-1ra/HA 100MG/KG Q2D
IL-1ra/HA 100MG/KG Q3D
TREATMENT
77.85%
62.06%
19.16%
MEAN ± S.E. PAW WEIGHT

FIG. 11A

```
   ATGGCTGCAGCTGAACCAAAATCTTCCGACAAAACTCACACATGCCCACCGTGCCCAGCA
 1 ------+---------+---------+---------+---------+---------+---  60
   M  A  A  A  E  P  K  S  S  D  K  T  H  T  C  P  P  C  P  A

   CCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTC
61 ------+---------+---------+---------+---------+---------+--- 120
   P  E  L  L  G  G  P  S  V  F  L  F  P  P  K  P  K  D  T  L

   ATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCT
121 ------+---------+---------+---------+---------+---------+--- 180
   M  I  S  R  T  P  E  V  T  C  V  V  V  D  V  S  H  E  D  P

   GAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCG
181 ------+---------+---------+---------+---------+---------+--- 240
   E  V  K  F  N  W  Y  V  D  G  V  E  V  H  N  A  K  T  K  P

   CGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAG
241 ------+---------+---------+---------+---------+---------+--- 300
   R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V  L  T  V  L  H  Q

   GACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCC
301 ------+---------+---------+---------+---------+---------+--- 360
   D  W  L  N  G  K  E  Y  K  C  K  V  S  N  K  A  L  P  A  P

   ATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTG
361 ------+---------+---------+---------+---------+---------+--- 420
   I  E  K  T  I  S  K  A  K  G  Q  P  R  E  P  Q  V  Y  T  L

   CCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGC
421 ------+---------+---------+---------+---------+---------+--- 480
   P  P  S  R  D  E  L  T  K  N  Q  V  S  L  T  C  L  V  K  G

   TTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTAC
481 ------+---------+---------+---------+---------+---------+--- 540
   F  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P  E  N  N  Y

   AAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACC
541 ------+---------+---------+---------+---------+---------+--- 600
   K  T  T  P  P  V  L  D  S  D  G  S  F  F  L  Y  S  K  L  T

   GTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCT
601 ------+---------+---------+---------+---------+---------+--- 660
   V  D  K  S  R  W  Q  Q  G  N  V  F  S  C  S  V  M  H  E  A

   CTGCACAACCACTACACGCAGAAGAGCCTCTCGCTCAGCCCGGGTAAAATGCGACCGTCC
661 ------+---------+---------+---------+---------+---------+--- 720
   L  H  N  H  Y  T  Q  K  S  L  S  L  S  P  G  K  M  R  P  S

   GGCCGTAAGAGCTCCAAAATGCAGGCTTTCCGTATCTGGGACGTTAACCAGAAAACCTTC
721 ------+---------+---------+---------+---------+---------+--- 780
   G  R  K  S  S  K  M  Q  A  F  R  I  W  D  V  N  Q  K  T  F
```

FIG. 11B

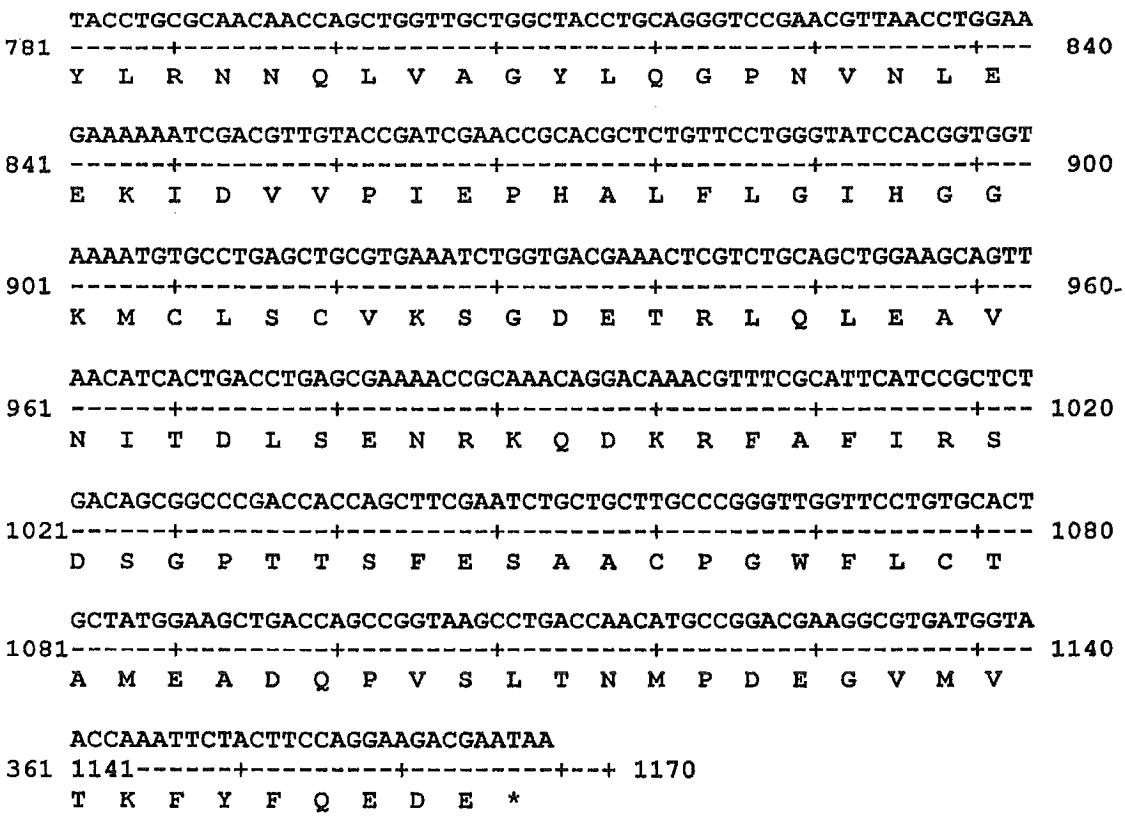

TACCTGCGCAACAACCAGCTGGTTGCTGGCTACCTGCAGGGTCCGAACGTTAACCTGGAA
781 ------+---------+---------+---------+---------+---------+--- 840
Y L R N N Q L V A G Y L Q G P N V N L E
GAAAAAATCGACGTTGTACCGATCGAACCGCACGCTCTGTTCCTGGGTATCCACGGTGGT
841 ------+---------+---------+---------+---------+---------+--- 900
E K I D V V P I E P H A L F L G I H G G
AAAATGTGCCTGAGCTGCGTGAAATCTGGTGACGAAACTCGTCTGCAGCTGGAAGCAGTT
901 ------+---------+---------+---------+---------+---------+--- 960
K M C L S C V K S G D E T R L Q L E A V
AACATCACTGACCTGAGCGAAAACCGCAAACAGGACAAACGTTTCGCATTCATCCGCTCT
961 ------+---------+---------+---------+---------+---------+--- 1020
N I T D L S E N R K Q D K R F A F I R S
GACAGCGGCCCGACCACCAGCTTCGAATCTGCTGCTTGCCCGGGTTGGTTCCTGTGCACT
1021------+---------+---------+---------+---------+---------+--- 1080
D S G P T T S F E S A A C P G W F L C T
GCTATGGAAGCTGACCAGCCGGTAAGCCTGACCAACATGCCGGACGAAGGCGTGATGGTA
1081------+---------+---------+---------+---------+---------+--- 1140
A M E A D Q P V S L T N M P D E G V M V
ACCAAATTCTACTTCCAGGAAGACGAATAA
361 1141------+---------+---------+--+ 1170
T K F Y F Q E D E *

FIG. 12A

```
    ATGCGACCTCCGGCCGTAAGAGCTCCAAAATGCAGGCTTTCCGTATCTGGGACGTTAAC
  1 ------+---------+---------+---------+---------+---------+---   60
    M  R  P  S  G  R  K  S  S  K  M  Q  A  F  R  I  W  D  V  N

    CAGAAAACCTTCTACCTGCGCAACAACCAGCTGGTTGCTGGCTACCTGCAGGGTCCGAAC
 61 ------+---------+---------+---------+---------+---------+---  120
    Q  K  T  F  Y  L  R  N  N  Q  L  V  A  G  Y  L  Q  G  P  N

    GTTAACCTGGAAGAAAAAATCGACGTTGTACCGATCGAACCGCACGCTCTGTTCCTGGGT
121 ------+---------+---------+---------+---------+---------+---  180
    V  N  L  E  E  K  I  D  V  V  P  I  E  P  H  A  L  F  L  G

    ATCCACGGTGGTAAAATGTGCCTGAGCTGCGTGAAATCTGGTGACGAAACTCGTCTGCAG
181 ------+---------+---------+---------+---------+---------+---  240
    I  H  G  G  K  M  C  L  S  C  V  K  S  G  D  E  T  R  L  Q

    CTGGAAGCAGTTAACATCACTGACCTGAGCGAAAACCGCAAACAGGACAAACGTTTCGCA
241 ------+---------+---------+---------+---------+---------+---  300
    L  E  A  V  N  I  T  D  L  S  E  N  R  K  Q  D  K  R  F  A

    TTCATCCGCTCTGACAGCGGCCCGACCACCAGCTTCGAATCTGCTGCTTGCCCGGGTTGG
301 ------+---------+---------+---------+---------+---------+---  360
    F  I  R  S  D  S  G  P  T  T  S  F  E  S  A  A  C  P  G  W

    TTCCTGTGCACTGCTATGGAAGCTGACCAGCCGGTAAGCCTGACCAACATGCCGGACGAA
361 ------+---------+---------+---------+---------+---------+---  420
    F  L  C  T  A  M  E  A  D  Q  P  V  S  L  T  N  M  P  D  E

    GGCGTGATGGTAACCAAATTCTACTTCCAGGAAGACGAAGCTGCAGCTGAACCAAAATCT
421 ------+---------+---------+---------+---------+---------+---  480
    G  V  M  V  T  K  F  Y  F  Q  E  D  E  A  A  A  E  P  K  S

    TCCGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGGACCGTCA
481 ------+---------+---------+---------+---------+---------+---  540
    S  D  K  T  H  T  C  P  P  C  P  A  P  E  L  L  G  G  P  S

    GTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTC
541 ------+---------+---------+---------+---------+---------+---  600
    V  F  L  F  P  P  K  P  K  D  T  L  M  I  S  R  T  P  E  V

    ACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTG
601 ------+---------+---------+---------+---------+---------+---  660
    T  C  V  V  V  D  V  S  H  E  D  P  E  V  K  F  N  W  Y  V

    GACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACG
661 ------+---------+---------+---------+---------+---------+---  720
    D  G  V  E  V  H  N  A  K  T  K  P  R  E  E  Q  Y  N  S  T
```

FIG. 12B

```
    TACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC
721 ------+---------+---------+---------+---------+---------+---  780
    Y  R  V  V  S  V  L  T  V  L  H  Q  D  W  L  N  G  K  E  Y

    AAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCC
781 ------+---------+---------+---------+---------+---------+---  840
    K  C  K  V  S  N  K  A  L  P  A  P  I  E  K  T  I  S  K  A

    AAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACC
841 ------+---------+---------+---------+---------+---------+---  900
    K  G  Q  P  R  E  P  Q  V  Y  T  L  P  P  S  R  D  E  L  T

    AAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTG
901 ------+---------+---------+---------+---------+---------+---  960
    K  N  Q  V  S  L  T  C  L  V  K  G  F  Y  P  S  D  I  A  V

    GAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGAC
961 ------+---------+---------+---------+---------+---------+--- 1020
    E  W  E  S  N  G  Q  P  E  N  N  Y  K  T  T  P  P  V  L  D

    TCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAG
1021------+---------+---------+---------+---------+---------+--- 1080
    S  D  G  S  F  F  L  Y  S  K  L  T  V  D  K  S  R  W  Q  Q

    GGGAACGTCTTCTCATGCTCCGTGTGCATGAGGCTCTGCACAACCACTACACGCAGAAG
1081------+---------+---------+---------+---------+---------+--- 1140
    G  N  V  F  S  C  S  V  M  H  E  A  L  H  N  H  Y  T  Q  K

    AGCCTCTCGCTCAGCCCGGGTAAATAA
1141------+---------+---------- 1167
    S  L  S  L  S  P  G  K  *
```

COMPOSITION AND METHOD FOR TREATING INFLAMMATORY DISEASES

This application claims priority of provisional application Ser. No. 60/055,185, filed Aug. 8, 1997.

FIELD OF THE INVENTION

The present invention relates to the field of inflammation. More specifically, the present invention relates to a composition for the purpose of preventing or treating inflammatory diseases.

BACKGROUND OF THE INVENTION

Inflammation is the body's defense reaction to injuries such as those caused by mechanical damage, infection or antigenic stimulation. An inflammatory reaction may be expressed pathologically when inflammation is induced by an inappropriate stimulus such as an autoantigen, is expressed in an exaggerated manner or persists well after the removal of the injurious agents.

While the etiology of inflammation is poorly understood, considerable information has recently been gained regarding the molecular aspects of inflammation. This research has led to identification of certain cytokines which are believed to figure prominently in the mediation of inflammation. Cytokines are extracellular proteins that modify the behavior of cells, particularly those cells that are in the immediate area of cytokine synthesis and release. Interleukin-1 (IL-1) is one of the most potent inflammatory cytokines yet discovered and a cytokine which is thought to be a key mediator in many diseases and medical conditions, termed "interleukin-1 mediated diseases". IL-1, which is manufactured (though not exclusively) by cells of the macrophage/monocyte lineage, may be produced in two forms: IL-1 alpha (IL-1α) and IL-1 beta (IL-1β).

A disease or medical condition is considered to be an "interleukin-1 mediated disease" if the spontaneous or experimental disease or medical condition is associated with elevated levels of IL-1 in bodily fluids or tissue or if cells or tissues taken from the body produce elevated levels of IL-1 in culture. In many cases, such interleukin-1 mediated diseases are also recognized by the following additional two conditions: (1) pathological findings associated with the disease or medical condition can be mimicked experimentally in animals by the administration of IL-1; and (2) the pathology induced in experimental animal models of the disease or medical condition can be inhibited or abolished by treatment with agents which inhibit the action of IL-1. In most interleukin-1 mediated diseases at least two of the three conditions are met, and in many interleukin-1 mediated diseases all three conditions are met. A non-exclusive list of acute and chronic interleukin-1 (IL-1)-mediated inflammatory diseases includes but is not limited to the following: acute pancreatitis; ALS; Alzheimer's disease; cachexia/anorexia; asthma; atherosclerosis; chronic fatigue syndrome, fever; diabetes (e.g., insulin diabetes); glomerulonephritis; graft versus host rejection; hemohorragic shock; hyperalgesia, inflammatory bowel disease; inflammatory conditions of a joint, including osteoarthritis, psoriatic arthritis and rheumatoid arthritis; ischemic injury, including cerebral ischemia (e.g., brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration); lung diseases (e.g., ARDS); multiple myeloma; multiple sclerosis; myelogenous (e.g., AML and CML) and other leukemias; myopathies (e.g., muscle protein metabolism, esp. in sepsis); osteoporosis; Parkinson's disease; pain; pre-term labor; psoriasis; reperfusion injury; septic shock; side effects from radiation therapy, temporal mandibular joint disease, tumor metastasis; or an inflammatory condition resulting from strain, sprain, cartilage damage, trauma, orthopedic surgery, infection or other disease processes.

Inflammatory conditions of a joint are chronic joint diseases that afflict and disable, to varying degrees, millions of people worldwide. Rheumatoid arthritis is a disease of articular joints in which the cartilage and bone are slowly eroded away by a proliferative, invasive connective tissue called pannus, which is derived from the synovial membrane. The disease may involve peri-articular structures such as bursae, tendon sheaths and tendons as well as extra-articular tissues such as the subcutis, cardiovascular system, lungs, spleen, lymph nodes, skeletal muscles, nervous system (central and peripheral) and eyes (Silberberg (1985), *Anderson's Pathology,* Kissane (ed.), II:1828). Osteoarthritis is a common joint disease characterized by degenerative changes in articular cartilage and reactive proliferation of bone and cartilage around the joint. Osteoarthritis is a cell-mediated active process that may result from the inappropriate response of chondrocytes to catabolic and anabolic stimuli. Changes in some matrix molecules of articular cartilage reportedly occur in early osteoarthritis (Thonar et al. (1993), *Rheumatic disease clinics of North America,* Moskowitz (ed.), 19:635–657 and Shinmei et al. (1992), *Arthritis Rheum.,* 35:1304–1308).

It is believed that rheumatoid arthritis results from the presentation of a relevant antigen to an immunogenetically susceptible host. The antigens that could potentially initiate an immune response that results in rheumatoid arthritis might be endogenous or exogenous. Possible endogenous antigens include collagen, mucopolysaccharides and rheumatoid factors. Exogenous antigens include mycoplasms, mycobacteria, spirochetes and viruses. By-products of the immune reaction inflame the synovium (i.e., prostaglandins and oxygen radicals) and trigger destructive joint changes (i.e., collagenase).

There is a wide spectrum of disease severity, but many patients run a course of intermittent relapses and remissions with an overall pattern of slowly progressive joint destruction and deformity. The clinical manifestations may include symmetrical polyarthritis of peripheral joints with pain, tenderness, swelling and loss of function of affected joints, morning stiffness, and loss of cartilage, erosion of bone matter and subluxation of joints after persistent inflammation. Extra-articular manifestations include rheumatoid nodules, rheumatoid vasculitis, pleuropulmonary inflammations, scleritis, sicca syndrome, Felty's syndrome (splenomegaly and neutropenia), osteoporosis and weight loss (Katz (1985), *Am. J. Med.,* 79:24 and Krane and Simon (1986), *Advances in Rheumatology,* Synderman (ed.), 70(2):263–284). The clinical manifestations result in a high degree of morbidity resulting in disturbed daily life of the patient.

The involvement of interleukin-1 in arthritis has been implicated by two distinct lines of evidence. First, increased levels of interleukin-1, and of the mRNA encoding it, have been found in the synovial tissue and fluid of arthritic joints. See, for example, Buchan et al., "Third Annual General Meeting of the British Society for Rheumatology," London, England, Nov. 19–21, 1988, *J. Rheumatol.,* 25(2); Fontana et al. (1982), *Rheumatology int.,* 2:49–53; and Duff et al. (1988), *Monokines and Other Non-Lymphocytic Cytokines,* M. Powanda et al.(eds), pp. 387–392 (Alan R. Liss, Inc.).

Second, the administration of interleukin-1 to healthy joint tissue has been shown on numerous occasions to result in the erosion of cartilage and bone. In one experiment, intraarticular injections of IL-1 into rabbits were shown to cause cartilage destruction in vivo (Pettipher et al. (1986), *Proc. Nat'l Acad. Sci. U.S.A.,* 83:8749–8753). In other studies, IL-1 was shown to cause the degradation of both cartilage and bone in tissue explants (Saklatavala et al. (1987), *Development of Diseases of Cartilage and Bone Matrix,* Sen and Thornhill (eds.), pp. 291–298 (Alan R. Liss, Inc.) and Stashenko et al. (1987), *The American Association of Immunologists,* 183:1464–1468). One generally accepted theory which is used to explain the causal link between IL-1 and arthritis is that IL-1 stimulates various cell types, such as fibroblasts and chondrocytes, to produce and secrete proinflammatory or degradative compounds such as prostaglandin E_2 and metalloproteinases.

Interleukin-1 receptor antagonist (IL-1ra) is a human protein that acts as a natural inhibitor of interleukin-1. IL-1 receptor antagonist (IL-1ra) has been disclosed as a potential agent for use in the clinical treatment of IL-1-mediated diseases (Australian Patent No. 649245). However, IL-1ra has a relatively short half-life. It therefore would be advantageous to administer IL-1ra in a manner which maintains a preselected concentration range of IL-1ra in the blood stream (e.g., controlled release formulations, Fc fusion proteins and chemical attachment, and continuous pump infusion).

With the advances in recombinant DNA technologies, the availability of recombinant proteins for therapeutic use has engendered advances in protein formulation and chemical modification. A review article describing protein modification and fusion proteins is Francis, *Focus on Growth Factors* 3:4–10 (May 1992) (published by Mediscript, Mountview Court, Friern Barnet Lane, London N20, OLD, UK).

One such modification is the use of the Fc region of immunoglobulins. Antibodies comprise two functionally independent parts, a variable domain known as "Fab", which binds antigen, and a constant domain, known as "Fc" which provides the link to effector functions such as complement or phagocytic cells. The Fc portion of an immunoglobulin has a long plasma half-life, whereas the Fab is short-lived (Capon, et al. (1989), *Nature,* 337:525–531).

Therapeutic protein products have been constructed using the Fc domain to provide longer half-life or to incorporate functions such as Fc receptor binding, protein A binding, complement fixation and placental transfer which all reside in the Fc proteins of immunoglobulins. Id. For example, the Fc region of an IgG1 antibody has been fused to the N-terminal end of CD30-L, a molecule which binds CD30 receptors expressed on Hodgkin's Disease tumor cells, anaplastic lymphoma cells, T-cell leukemia cells and other malignant cell types (U.S. Pat. No. 5,480,981). IL-10, an anti-inflammatory and antirejection agent has been fused to murine Fcγ2a in order to increase the cytokine's short circulating half-life. Zheng, X. et al. (1995), *The Journal of Immunology,* 154: 5590–5600. Studies have also evaluated the use of tumor necrosis factor receptor linked with the Fc protein of human IgG1 to treat patients with septic shock. Fisher, C. et al., *N. Engl. J. Med.,* 334: 1697–1702 (1996); Van Zee, K. et al., *The Journal of Immunology,* 156: 2221–2230 (1996) and rheumatoid arthritis (Moreland, et al. (1997), *N. Engl. J. Med.,* 337(3):141–147. Fc has also been fused with CD4 receptor to produce a therapeutic protein for treatment of AIDS (Capon et al. (1989), Nature, 337:525–531). In addition, the N-terminus of interleukin 2 has also been fused to the Fc portion of IgG1 or IgG3 to overcome the short half life of interleukin 2 and its systemic toxicity (Harvill et al. (1995), Immunotechnology, 1: 95–105).

One material useful in controlled release formulations is hyaluronic acid. Hyaluronic acid is a naturally occurring mucopolysaccharide consisting of residues of D-glucoronic acid and N-acetyl-D-glucosamine in an unbranched chain. The polymer has an average molecular weight of $(5–6)\times10^6$ and exhibits excellent biocompatibility. In the articular cartilage, hyaluronic acid plays an important role in the construction of the cartilage matrix by aggregating with proteoglycan. Furthermore, it has been reported that under pathological conditions such as rheumatoid arthritis, osteoarthritis and infectious arthritis, the concentrations and molecular weight of hyaluronic acid in the joint are changed and cause changes in the nature of the synovial fluid.

Both chemical cross-linking and derivatization of hyaluronic acid have been used to enhance its Theological properties or increase the degradation time of certain drugs (Cortivo et al. (1991), *Biomaterials,* 2:727–730; Benedetti et al. (1990), *J. Controlled Release,* 13:33–41 and Hunt et al. (1990), *J. Controlled Release,* 12:159–169).

It has been shown that the injection of high molecular weight hyaluronic acid derivatives may restore the damaged hyaluronic acid layer on the articular cartilage surface and may be effective for treating some kinds of articular conditions in clinical and fundamental tests. Examples of scientific publications describing such use of hyaluronic acid derivatives for treatment of articular conditions include Nizolek & White (1981), *Cornell Vet.,* 71:355–375; Namiki et al. (1982), *Int. J. Chem. Pharmacol., Therapy and Toxicol.,* 20:501–507; Asheim and Lindblad (1976), *Acta Vet Scand,* 17 (4):379–394; Svanstrom (1978), *Proceedings of the* 24th *Annual Convention of the American Association of Equine Practitioners,* St Louis, Mo., p. 345–348; Wigren et al. (1975), *Upsala J Med Sci Suppl,* 17:1–20; and Gingerich et al. (1980), *Res Vet Sci,* 30:192–197. The use of hyaluronic acid in human joints is reported by Peyron et al. (1974), *Pathologie Biologie,* 22(8):731–736. The intraarticular use of hyaluronic acid in horse joints has been commercially promoted in connection with Pharmacia's Hylartil™ and Hylartin V™• products and Sterivet's Synacid™ product. However, although symptoms such as pain and stiffness become a serious problem in the treatment of joint diseases, hyaluronic acid does not directly improve such symptoms.

Additionally, hyaluronic acid has been used for drug delivery. One scientific publication describes the use of hyaluronic acid both alone and with cortisone in various animal joints, especially horses, is Rydell et al. (1971), *Clinical Orthopaedics and Related Research,* 80:25–32. Another scientific publication describes the preparation of microspheres from hyaluronic acid esters were used for the nasal delivery of insulin (Illum et al. (1994), *J. Controlled Release,* 29:133–141). Blank spheres were prepared by an emulsification/solvent evaporation technique, exposed to an insulin solution for an hour, and then lyophilized. When administered to sheep, the mean bioavailability was found to be 11% when compared with insulin administered by the subcutaneous route. This system has also been used as a deliverv device for nerve growth factor (Ghezzo et al. (1992), *Int. J. Pharm.,* 87:21–29). However, it has been reported that when dog knees were injected with a physiological concentration (3 mg/ml) of high molecular weight (Mr 6×10^6) or low molecular weight (Mr 5×10^5) hyaluronic acid mixed with radioactive albumin, the albumin distribution volume and clearance rate slightly exceeded those in knees in which the concentration (0.03 mg/ml) of high molecular weight hyaluronic acid or the concentration (0.3 mg/ml) low molecular weight hyaluronic acid was reduced (Myers and Brandt (1995), *J. Rheumatol.,* 22:1732–1739). This reference suggests that a combination of hyaluronic acid with a protein, such as IL-1ra, would be no more effective than hyaluronic acid alone in the treatment of inflammatory diseases, particularly when administered via intraarticular injection.

Due to the identification of the IL-1ra protein as a promising therapeutic protein, there exists a need to develop IL-1ra compositions where protein formulations and chemical modifications achieve decreased protein degradation, increased stability and circulation time. The present invention provides such compositions.

It is an objective of the present invention to provide therapeutic methods and compositions for the treatment of IL-1-mediated inflammatory diseases. This and other objects of the present invention will become apparent from the description hereinafter.

SUMMARY OF THE INVENTION

The present invention stems from the observation that continuous presence in the bloodstream, in predictable amounts based on a determined dosage regimen, of a proteinaceous IL-1 inhibitor, e.g., IL-1ra, by extended delivery means, e.g., controlled release polymer formulations (e.g., hyaluronan), IL-1ra fusion proteins and chemical attachment, and continuous pump infusion, results in improved treatment of IL-1-mediated inflammatory diseases. The type of treatment herein referred to is intended for mammals, including humans.

BRIEF DESCRIPTION OF THE FIGURES

Numerous aspects and advantages of the present invention will become apparent upon review of the figures, wherein:

FIG. **1** shows the serum levels of IL-1ra after subcutaneous injection of either IL-1ra in citrate buffer (CSEP) alone or IL-1ra mixed with hyaluronic acid in CSEP.

FIG. **2** shows the amount of IL-1ra remaining in guinea pig joints after intraarticular injection of either IL-1ra in CSEP alone or IL-1ra mixed with hyaluronic acid in CSEP.

FIG. **3** shows the concentration of IL-1ra in recovered synovial fluid of rabbits after intraarticular injection of either IL-1ra in CSEP alone or IL-1ra mixed with hyaluronic acid in CSEP.

FIG. **4** shows the histological evaluation of disease severity in knee joints of rats immunized with bovine type II collagen, after intraarticular injection of either hyaluronic acid in CSEP alone or IL-1ra mixed with hyaluronic acid in CSEP.

FIG. **5** shows a nucleic acid sequence (SEQ ID NO:1) encoding recombinant human IL-1ra (rhuIL-1ra). Also shown is the amino acid sequence (SEQ ID NO:2) of rhuIL-1ra, with the initial amino acid being M_n wherein n equal 0 or 1.

FIG. **6**. shows the effects of once daily injection (QD) of IL-1ra mixed with hyaluronic acid in CSEP shown in comparison to IL-1ra in CSEP or hyaluronic acid in CSEP or CSEP alone on ankle joint diameter over time in rats with established type II collagen arthritis.

FIG. **7**. shows the effects of once daily injection (QD) of IL-1ra mixed with hyaluronic acid in CSEP shown in comparison to IL-1ra in CSEP or hyaluronic acid in CSEP or CSEP alone on final paw weights in rats with established type II collagen arthritis.

FIG. **8**. shows the effects of once daily injection (QD) of IL-1ra mixed with hyaluronic acid in CSEP shown in comparison to IL-1ra in CSEP or hyaluronic acid in CSEP or CSEP alone on inflammation, pannus formation, and cartilage and bone damage in rats with established type II collagen arthritis.

FIG. **9**. shows the effects of once daily injection (QD), every other day injection (Q2D) or every third day injection (Q3D) of IL-1ra mixed with hyaluronic acid in CSEP shown in comparison to hyaluronic acid in CSEP (QD) or no treatment on ankle joint diameter over time in rats with established type II collagen arthritis.

FIG. **10**. shows the effects of once daily injection (QD), every other day injection (Q2D) or every third day injection (Q3D) of IL-1ra mixed with hyaluronic acid in CSEP shown in comparison to hyaluronic acid in CSEP (QD) or no treatment on final paw weight in rats with established type II collagen arthritis.

FIG. **11** shows a nucleic acid sequence (SEQ ID NO:13) encoding recombinant human IL-1ra-Fc fusion protein ("rhuIL-1ra-Fc"). Also shown is the amino acid sequence (SEQ ID NO:14) of rhuIL-1ra-Fc.

FIG. **12** shows a nucleic acid sequence (SEQ ID NO:15) encoding Fc-recombinant human IL-1ra ("Fc-rhuIL-1ra"). Also shown is the amino acid sequence (SEQ ID NO:16) of Fc-rhuIL-1ra.

DETAILED DESCRIPTION

Interleukin-1 inhibitors may be from any protein capable of specifically preventing activation of cellular receptors to IL-1. Classes of interleukin-1 inhibitors include: interleukin-1 receptor antagonists such as IL-1ra as

described below; anti-IL-1 receptor monoclonal antibodies, e.g., EP 623674; Il-1 binding proteins such as soluble IL-1 receptors, e.g., U.S. Pat. Nos. 5,492,888, 5,488,032, 5,464, 937, 5,319,071 and 5,180,812; anti-IL-1 monoclonal antibodies, e.g., WO 9501997, WO 9402627, WO 9006371, U.S. Pat. No. 4,935,343, EP 364778, EP 267611 and EP 220063; and IL-1 receptor accessory proteins, e.g., WO 96/23067, the disclosures of which are incorporated herein by reference.

Interleukin-1 receptor antagonist (IL-1ra) is a human protein that acts as a natural inhibitor of interleukin-1. Preferred receptor antagonists, as well as methods of making and using thereof, are described in U.S. Pat. No. 5,075,222 (referred to herein as the '222 patent); WO 91/08285; WO 91/17184; AU 9173636; WO 92/16221; WO 93/21946; WO 94/06457; WO 94/21275; FR 2706772; WO 94/21235; DE 4219626, WO 94/20517; WO 96/22793, WO 97/28828 and WO 98/24477, the disclosures of which are incorporated herein by reference.

Specifically, three useful forms of IL-1ra and variants thereof are disclosed and described in the '222 patent. The first of these, IL-1raα, is characterized as a 22–23 kD molecule on SDS-PAGE with an approximate isoelectric point of 4.8, eluting from a Mono Q FPLC column at around 52 mM NaCl in Tris buffer, pH 7.6. The second, IL-1raβ, is characterized as a 22–23 kD protein, eluting from a Mono Q column at 48 mM NaCl. Both IL-1raα and IL-1raβ are glycosylated. The third, IL-1rax, is characterized as a 20 kD protein, eluting from a Mono Q column at 48 mM NaCl, and is non-glycosylated. All three of these inhibitors possess similar functional and immunological activities.

Methods for producing IL-1ra are also disclosed in the '222 patent. One disclosed method consists of isolating the IL-1ra from human monocytes, where they are naturally produced. A second disclosed method involves isolating the gene responsible for coding IL-1ra, cloning the gene in suitable vectors and cells types, expressing the gene to produce the inhibitors and harvesting the inhibitors. The latter method, which is exemplary of recombinant DNA methods in general, is a preferred method. Recombinant DNA methods are preferred in part because they are capable of achieving comparatively greater amounts of protein at greater purity. Thus, the invention also encompasses IL-1ra containing an N-terminal methionyl group as a consequence of expression in prokaryotic cells, such as *E. coli.*

As stated above, the present invention also includes modified forms of IL-1ra. The modified forms of IL-1ra as used herein include variant polypeptides in which amino acids have been (1) deleted from ("deletion variants"), (2) inserted into ("addition variants") or (3) substituted for ("substitution variants") residues within the amino acid sequence of IL-1ra.

For IL-1ra deletion variants, each polypeptide may typically have an amino sequence deletion ranging from about 1 to 30 residues, more typically from about 1 to 10 residues and most typically from about 1 to 5 contiguous residues. N-terminal, C-terminal and internal intrasequence deletions are contemplated. Deletions within the IL-1ra amino acid sequence may be made in regions of low homology with the sequences of other members of the IL-1 family. Deletions within the IL-1ra amino acid sequence may be made in areas of substantial homology with the sequences of other members of the IL-1 family and will be more likely to significantly modify the biological activity.

For IL-1ra addition variants, each polypeptide may include an amino- and/or carboxyl-terminal fusion ranging in length from one residue to one hundred or more residues, as well as internal intrasequence insertions of single or multiple amino acid residues. Internal additions may range typically from about 1 to 10 amino acid residues, more typically from about 1 to 5 amino acid residues and most typically from about 1 to 3 amino acid residues.

Amino-terminus addition variants include the addition of a methionine (for example, as an artifact of the direct expression of the protein in bacterial recombinant cell culture) or an additional amino acid residue or sequence. A further example of an amino-terminal insertion includes the fusion of a signal sequence, as well as or with other pre-pro sequences, to facilitate the secretion of protein from recombinant host cells. Each polypeptide may comprise a signal sequence selected to be recognized and processed, i.e., cleaved by a signal peptidase, by the host cell. For prokaryotic host cells that do not recognize and process the native IL-1ra signal sequence, each polypeptide may comprise a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase or heat-stable enterotoxin II leaders. For yeast cells, each polypeptide may have a signal sequence selected, for example, from the group of the yeast invertase, alpha factor or acid phosphatase leader sequences. For mammalian cell expression, each polypeptide may have the native signal sequence of IL-1ra, although other mammalian signal sequences may be suitable, for example, sequences derived from other IL-1 family members.

Amino- and carboxy-terminus addition variants include chimeric proteins wherein each comprises the fusion of IL-1ra with all or part of a constant domain of a heavy or light chain of human immunoglobulin (Ellison, J. W. et al. (1982), *Nucleic Acids Res.,* 10:4071–4079) at the amino-terminus (e.g., Fc-rhuIL-1ra), the carboxy-terminus (e.g., rhuIL-1ra-Fc) or both (collectively, "rhuIL-1ra Fc fusion proteins"). Such chimeric proteins are preferred wherein the immunoglobulin portion of each comprises all domains except the first domain of the constant region of the heavy chain of human immunoglobulin, such as IgG, IgA, IgM or IgE (especially IgG, e.g., IgG1 or IgG3). A skilled artisan will appreciate that any amino acid of each immunoglobulin portion can be deleted or substituted with one or more amino acids, or one or more amino acids can be added as long as the IL-1ra still antagonizes the IL-1 receptor, and the immunoglobulin portion shows one or more of its characteristic properties.

Modifications may be made to introduce four amino acid substitutions to ablate the Fc receptor binding site and the complement (C1q) binding site.

Likewise, one or more tyrosine residues can be replaced by phenyalanine residues as well. In addition, other variant amino acid insertions, deletions and/or substitutions are also contemplated and are within the scope of the present invention. Furthermore, alterations may be in the form of altered amino acids, such as peptidomimetics or D-amino acids. The Fc protein may be also linked to the IL-1ra proteins by

"linker" moieties whether chemical or amino acids of varying lengths. Such chemical linkers are well known in the art.

For IL-1ra substitution variants, each such polypeptide may have at least one amino acid residue in IL-1ra removed and a different residue inserted in its place. Substitution variants include allelic variants, which are characterized by naturally-occurring nucleotide sequence changes in the species population that may or may not result in an amino acid change. One skilled in the art can use any information known about the binding or active site of the polypeptide in the selection of possible mutation sites. Exemplary substitution variants are taught in WO 91/17184, WO 92/16221, and WO 96/09323.

One method for identifying amino acid residues or regions for mutagenesis of a protein is called "alanine scanning mutagenesis" (Cunningham and Wells (1989), *Science,* 244:1081–1085, the disclosure of which is hereby incorporated by reference). In this method, an amino acid residue or group of target residues of a protein is identified (e.g., charged residues such as Arg, Asp, His, Lys and Glu) and replaced by a neutral or negatively-charged amino acid (most preferably alanine or polyalanine) to effect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those residues demonstrating functional sensitivity to the substitutions are then refined by introducing additional or alternate residues at the sites of substitution. Thus, the site for introducing an amino acid sequence modification is predetermined and, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted and the resulting variant polypeptide screened for the optimal combination of desired activity and degree of activity.

The sites of greatest interest for substitutional mutagenesis include sites where the amino acids found in IL-ra are substantially different in terms of side-chain bulk, charge and/or hydrophobicity from IL-1ra-like proteins such as IL-1ra's of other various species or of other members of the IL-1 family. Other sites of interest include those in which particular residues of IL-1ra are identical with those of such IL-1ra-like proteins. Such positions are generally important for the biological activity of a protein. Initially, these sites are modified by substitution in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of "Preferred Substitutions". If such substitutions result in a change in biological activity, then more substantial changes (Exemplary Substitutions) are introduced and/or other additions/deletions may be made and the resulting polypeptides screened.

TABLE 1

	Amino Acid Substitutions	
Original Residue	Preferred Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Lys; Arg
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn

TABLE 1-continued

	Amino Acid Substitutions	
Original Residue	Preferred Substitutions	Exemplary Substitutions
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Arg	Asn; Gln; Lys; Arg
Ile (I)	Leu	Leu; Val; Met; Ala; Phe; norleucine
Leu (L)	Ile	norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Leu	Leu; Val; Ile; Ala
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; norleucine

In making such changes of an equivalent nature, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (−0.4); threonine (−0.7); serine (−0.8); tryptophan (−0.9); tyrosine (−1.3); proline (−1.6); histidine (−3.2); glutamate (−3.5); glutamine (−3.5); aspartate (−3.5); asparagine (−3.5); lysine (−3.9); and arginine (−4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte and Doolittle (1982), *J. Mol. Biol.,* 157:105–131, the disclosure of which are incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biological functional equivalent protein or peptide thereby created is intended for use in immunological embodiments, as in the present case.

U.S. Pat. No. 4,554,101, the disclosure of which are incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e. with a biological property of the protein.

As detailed in U.S. Pat. No. 4,554,101, the following hydrophilicity values have been assigned to amino acid

residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0±1); glutamate (+3.0±1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (−0.4); proline (−0.5+1); alanine (−0.5); histidine (−0.5); cysteine (−1.0); methionine (−1.3); valine (−1.5); leucine (−1.8); isoleucine (−1.8); tyrosine (−2.3); phenylalanine (−2.5); tryptophan (−3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

U.S. Pat. No. 4,554,101 also teaches the identification and preparation of epitopes from primary amino acid sequences on the basis of hydrophilicity. Through the methods disclosed in U.S. Pat. No. 4,554,101 one of skill in the art would be able to identify epitopes from within an amino acid sequence such as the IL-ra sequences disclosed herein. These regions are also referred to as "epitopic core regions".

Numerous scientific publications have been devoted to the prediction of secondary structure, and to the identification of epitopes, from analyses of amino acid sequences (Chou and Fasman (1974), *Biochemistry,* 13(2):222–245; Chou and Fasman, *Biochemistry,* 113(2):211–222; Chou and Fasman (1978), *Adv. Enzymol. Relat. Areas Mol. Biol.,* 47:45–148; Chou and Fasman, *Ann. Rev. Biochem.,* 47:251–276 and Chou and Fasman (1979), *Biophys. J.,* 26:367–384, the disclosures of which are incorporated herein by reference). Moreover, computer programs are currently available to assist with predicting antigenic portions and epitopic core regions of proteins. Examples include those programs based upon the Jameson-Wolf analysis (Jameson and Wolf (1988), *Comput. Appl. Biosci.,* 4(1):181–186 and Wolf et al. (1988), *Comput. Appl. Biosci.,* 4(1):187–191, the disclosures of which are incorporated herein by reference), the program PepPlot® (Brutlag et al. (1990) *CABS,* 6:237–245 and Weinberger et al. (1985), *Science,* 228:740–742, the disclosures of which are incorporated herein by reference), and other new programs for protein tertiary structure prediction (Fetrow and Bryant (1993), *BIOTECHNOLOGY,* 11:479–483, the disclosure of which are incorporated herein by reference).

Conservative modifications to the amino acid sequence (and the corresponding modifications to the encoding nucleic acid sequence) of IL-1ra are expected to produce proteins having similar functional and chemical characteristics. In contrast, substantial modifications in the functional and/or chemical characteristics of IL-1ra may be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the protein at the target site or (c) the bulk of the side chain. Naturally-occurring residues are divided into groups based on common side chain properties:

1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
2) neutral hydrophilic: Cys, Ser, Thr;
3) acidic: Asp, Glu;
4) basic: Asn, Gln, His, Lys, Arg;
5) aromatic: Trp, Tyr, Phe; and
6) residues that influence chain orientation: Gly, Pro.

Non-conservative substitutions may involve the exchange of a member of one of these groups for another. Such substituted residues may be introduced into regions of IL-1ra that are homologous or non-homologous with other IL-1 family members.

Specific mutations in the sequence of IL-1ra may involve substitution of a non-native amino acid at the N-terminus, C-terminus or at any site of the protein that is modified by the addition of an N-linked or O-linked carbohydrate. Such modifications may be of particular utility, such as in the addition of an amino acid (e.g., cysteine), which is advantageous for the linking of a water soluble polymer to form a derivative, as described below. Further, the sequence of IL-1ra may be modified to add glycosylation sites or to delete N-linked or O-linked glycosylation sites. An asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either Asn-Xaa-Thr or Asn-Xaa-Ser, where Xaa can be any amino acid other than Pro.

In a specific embodiment, the variants are substantially homologous to the amino acid of IL-1ra (SEQ ID NO:2). The term "substantially homologous" as used herein means a degree of homology that is preferably in excess of 70%, more preferably in excess of 80%, even more preferably in excess of 90% or most preferably even 95%. The percentage of homology as described herein is calculated as the percentage of amino acid residues found in the smaller of the two sequences which align with identical amino acid residues in the sequence being compared when four gaps in a length of 100 amino acids may be introduced to assist in that alignment, as set forth by Dayhoff in *Atlas of Protein Sequence and Structure,* 5:124 (1972), National Biochemical Research Foundation, Washington, D.C., the disclosure of which is hereby incorporated by reference. Also included as substantially homologous are variants of IL-1ra which may be isolated by virtue of cross-reactivity with antibodies to the amino acid sequence of SEQ ID NO:2 or whose genes may be isolated through hybridization with the DNA of SEQ ID NO:1 or with segments thereof.

The production of variants of IL-1ra is described in further detail below. Such variants may be prepared by introducing appropriate nucleotide changes into the DNA encoding variants of IL-1ra or by in vitro chemical synthesis of the desired variants of IL-1ra. It will be appreciated by those skilled in the art that many combinations of deletions, insertions and substitutions can be made, provided that the final variants of IL-1ra are biologically active.

Mutagenesis techniques for the replacement, insertion or deletion of one or more selected amino acid residues are well known to one skilled in the art (e.g., U.S. Pat. No. 4,518,584, the disclosure of which is hereby incorporated by reference). There are two principal variables in the construction of each amino acid sequence variant, the location of the mutation site and the nature of the mutation. In designing each variant, the location of each mutation site and the nature of each mutation will depend on the biochemical characteristic(s) to be modified. Each mutation site can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections, depending upon the results achieved, (2) deleting

the target amino acid residue or (3) inserting one or more amino acid residues adjacent to the located site.

Chemically modified derivatives of IL-1ra and variants of IL-1ra may be prepared by one skilled in the art, given the disclosures herein. Conjugates may be prepared using glycosylated, non-glycosylated or de-glycosylated IL-1ra and variants of IL-1ra. Typically, non-glycosylated IL-1ra and variants of IL-1ra will be used. Suitable chemical moieties for derivatization of IL-1ra and variants of IL-1ra include water soluble polymers.

Water soluble polymers are desirable because the protein to which each is attached will not precipitate in an aqueous environment, such as a physiological environment. Preferably, the polymer will be pharmaceutically acceptable for the preparation of a therapeutic product or composition. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically and, if so, the desired dosage, circulation time and resistance to proteolysis.

Suitable, clinically acceptable, water soluble polymers include, but are not limited to, polyethylene glycol (PEG), polyethylene glycol propionaldehyde, copolymers of ethylene glycol/propylene glycol, monomethoxy-polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol (PVA), polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1, 3,6-trioxane, ethylene/maleic anhydride copolymer, poly (β-amino acids) (either homopolymers or random copolymers), poly(n-vinyl pyrrolidone)polyethylene glycol, polypropylene glycol homopolymers (PPG) and other polyakylene oxides, polypropylene oxide/ethylene oxide copolymers, polyoxyethylated polyols (POG) (e.g., glycerol) and other polyoxyethylated polyols, polyoxyethylated sorbitol, or polyoxyethylated glucose, colonic acids or other carbohydrate polymers, Ficoll or dextran and mixtures thereof.

As used herein, polyethylene glycol is meant to encompass any of the forms that have been used to derivatize other proteins, such as mono-(C1–C10) alkoxy- or aryloxy-polyethylene glycol. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water.

The IL-1ra protein, may be prepared by attaching polyaminoacids or branch point amino acids to the IL-1ra protein. For example, the polyaminoacid may be a carrier protein which serves to increase the circulation half life of the protein (i.e., in addition to the advantages achieved via IL-1ra fusion protein above). For the present therapeutic purpose of the present invention, such polyaminoacids should be those which have or do not create neutralizing antigenic response, or other adverse responses. Such polyaminoacids may be selected from the group consisting of serum album (such as human serum albumin), an additional antibody or portion thereof (e.g. the Fc region), or other polyaminoacids, e.g. lysines. As indicated below, the location of attachment of the polyaminoacid may be at the N-terminus, or C-terminus, or other places in between, and also may be connected by a chemical "linker" moiety to IL-1ra.

The water soluble polymers each may be of any molecular weight and may be branched or unbranched. The water soluble polymers each typically have an average molecular weight of between about 2 kDa to about 100 kDa (the term "about" indicating that in preparations of a water soluble polymer, some molecules will weigh more, some less, than the stated molecular weight). The average molecular weight of each water soluble polymer preferably is between about 5 kDa and about 50 kDa, more preferably between about 12 kDa and about 25 kDa and most preferably about 20 kDa. Generally, the higher the molecular weight or the more branches, the higher the polymer:protein ratio. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release; the effects, if any, on biological activity; the ease in handling; the degree or lack of antigenicity and other known effects of a water soluble polymer on a therapeutic protein).

The water soluble polymers each should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. In general, chemical derivatization may be performed under any suitable condition used to react a protein with an activated polymer molecule. Activating groups which can be used to link the polymer to the active moieties include the following: sulfone, maleimide, sulfhydryl, thiol, triflate, tresylate, azidirine, oxirane and 5-pyridyl.

The water soluble polymers each are generally attached to the protein at the α- or ε-amino groups of amino acids or a reactive thiol group, but it is also contemplated that a water soluble group could be attached to any reactive group of the protein which is sufficiently reactive to become attached to a water soluble group under suitable reaction conditions. Thus, a water soluble polymer may be covalently bound to a protein via a reactive group, such as a free amino or carboxyl group. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residue. Those having a free carboxyl group may include aspartic acid residues, glutamic acid residues and the C-terminal amino acid residue. Those having a reactive thiol group include cysteine residues.

Methods for preparing proteins conjugated with water soluble polymers will each generally comprise the steps of (a) reacting a protein with a water soluble polymer under conditions whereby the protein becomes attached to one or more water soluble polymers and (b) obtaining the reaction product. Reaction conditions for each conjugation may be selected from any of those known in the art or those subsequently developed, but should be selected to avoid or limit exposure to reaction conditions such as temperatures, solvents and pH levels that would inactivate the protein to be modified. In general, the optimal reaction conditions for the reactions will be determined case-by-case based on known parameters and the desired result. For example, the larger the ratio of water soluble polymer:protein conjugate, the greater the percentage of conjugated product. The optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) may be determined by factors such as the desired degree of derivatization (e.g., mono-, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched and the reaction conditions used. The ratio of water soluble polymer (e.g., PEG) to protein will generally range from 1:1 to 100:1. One or more purified conjugates may be prepared from each

mixture by standard purification techniques, including among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography and electrophoresis.

One may specifically desire an N-terminal chemically modified protein. One may select a water soluble polymer by molecular weight, branching, etc., the proportion of water soluble polymers to protein (or peptide) molecules in the reaction mix, the type of reaction to be performed, and the method of obtaining the selected N-terminal chemically modified protein. The method of obtaining the N-terminal chemically modified protein preparation (i.e., separating this moiety from other monoderivatized moieties if necessary) may be by purification of the N-terminal chemically modified protein material from a population of chemically modified protein molecules. Selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. For example, one may selectively attach a water soluble polymer to the N-terminus of the protein by performing the reaction at a pH which allows one to take advantage of the pKa differences between the ε-amino group of the lysine residues and that of the α-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the water soluble polymer may be of the type described above and should have a single reactive aldehyde for coupling to the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

The present invention specifically contemplates the chemically derivatized protein to include mono- or poly- (e.g., 2–4) PEG moieties. Pegylation may be carried out by any of the pegylation reactions known in the art. Methods for preparing a pegylated protein product will generally comprise the steps of (a) reacting a protein product with polyethylene glycol (such as a reactive ester or aldehyde derivative of PEG) under conditions whereby the protein becomes attached to one or more PEG groups and (b) obtaining the reaction product(s). In general, the optimal reaction conditions for the reactions will be determined case by case based on known parameters and the desired result.

There are a number of attachment methods available to those skilled in the art. See, for example, EP 0 401 384, the disclosure of which is hereby incorporated by reference; see also, Malik et al. (1992), *Exp. Hematol.,* 20:1028–1035; Francis (1992), *Focus on Growth Factors,* 3(2):4–10, (published by Mediscript, Mountain Court, Friern Barnet Lane, London N20 OLD, UK); EP 0 154 316; EP 0 401 384; WO 92/16221; WO 95/34326; and the other publications cited herein that relate to pegylation, the disclosures of which are hereby incorporated by reference.

The pegylation specifically may be carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule. Thus, protein products according to the present invention include pegylated proteins wherein the PEG group(s) is (are) attached via acyl or alkyl groups. Such products may be mono-pegylated or poly-pegylated (e.g., containing 2–6, and preferably 2–5, PEG groups). The PEG groups are generally attached to the protein at the α- or ε-amino groups of amino acids, but it is also contemplated that the PEG groups could be attached to any amino group attached to the protein which is sufficiently reactive to become attached to a PEG group under suitable reaction conditions.

Pegylation by acylation generally involves reacting an active ester derivative of polyethylene glycol (PEG) with the protein. For the acylation reactions, the polymer(s) selected should have a single reactive ester group. Any known or subsequently discovered reactive PEG molecule may be used to carry out the pegylation reaction. A preferred activated PEG ester is PEG esterified to N-hydroxysuccinimide (NHS). As used herein, "acylation" is contemplated to include, without limitation, the following types of linkages between the therapeutic protein and a water soluble polymer such as PEG: amide, carbamate, urethane, and the like (Chamow (1994), *Bioconjugate Chem.,* 5(2):133–140). Reaction conditions may be selected from any of those known in the pegylation art or those subsequently developed, but should avoid conditions such as temperature, solvent and pH that would inactivate the protein to be modified.

Pegylation by acylation will generally result in a poly-pegylated protein. Preferably, the connecting linkage will be an amide. Also preferably, the resulting product will be substantially only (e.g., >95%) mono, di- or tri-pegylated. However, some species with higher degrees of pegylation may be formed in amounts depending on the specific reaction conditions used. If desired, more purified pegylated species may be separated from the mixture (particularly unreacted species) by standard purification techniques, including among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography and electrophoresis.

Pegylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with the protein in the presence of a reducing agent. For the reductive alkylation reaction, the polymer(s) selected should have a single reactive aldehyde group. An exemplary reactive PEG aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1–C10 alkoxy or aryloxy derivatives thereof (U.S. Pat. No. 5,252,714).

Pegylation by alkylation can also result in poly-pegylated protein. In addition, one can manipulate the reaction conditions to substantially favor pegylation only at the amino group of the N-terminus of the protein (i.e., a mono-pegylated protein). In either case of monopegylation or polypegylation, the PEG groups are preferably attached to the protein via a —CH_2—NH— group. With particular reference to the —CH_2— group, this type of linkage is referred to herein as an "alkyl" linkage.

Reductive alkylation to produce a substantially homogeneous population of mono-polymer/protein product will generally comprise the steps of:

(a) reacting a protein with a reactive PEG molecule under reductive alkylation conditions, at a pH suitable to permit

selective modification of the α-amino group at the amino terminus of said protein; and

(b) obtaining the reaction product(s). Derivatization via reductive alkylation to produce a monopegylated product exploits pKa differences between the lysine amino groups and the α-amino group at the N-terminus (the pKa being the pH at which 50% of the amino groups are protonated and 50% are not).

The reaction is performed at a pH which allows one to take advantage of the pKa differences between the ϵ-amino groups of the lysine residues and that of the α-amino group of the N-terminal residue of the protein. In general, if the pH is lower, a larger excess of polymer to protein will be desired (i.e., the less reactive the N-terminal α-amino group, the more polymer needed to achieve optimal conditions). If the pH is higher, the polymer:protein ratio need not be as large (i.e., more reactive groups are available, so fewer polymer molecules are needed). For purposes of the present invention, the pH will generally fall within the range of 3–9, preferably 3–6. For the reductive alkylation, the reducing agent should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation. Suitable reducing agents may be selected from sodium borohydride, sodium cyanoborohydride, dimethylamine borane, trimethylamine borane and pyridine borane. A particularly suitable reducing agent is sodium cyanoborohydride. Other reaction parameters such as solvent, reaction times, temperatures and means of purification of products can be determined case-by-case, based on the published information relating to derivatization of proteins with water soluble polymers.

By such selective derivatization, attachment of a water soluble polymer (that contains a reactive group such as an aldehyde) to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. The preparation will typically be greater than 90% monopolymer/protein conjugate, and more typically greater than 95% monopolymer/protein conjugate, with the remainder of observable molecules being unreacted (i.e., protein lacking the polymer moiety).

The pegylation also may specifically be carried out via water soluble polymers having at least one reactive hydroxy group (e.g. polyethylene glycol) can be reacted with a reagent having a reactive carbonyl, nitrile or sulfone group to convert the hydroxyl group into a reactive Michael acceptor, thereby forming an "activated linker" useful in modifying various proteins to provide improved biologically-active conjugates. "Reactive carbonyl, nitrile or sulfone" means a carbonyl, nitrile or sulfone group to which a two carbon group is bonded having a reactive site for thiol-specific coupling on the second carbon from the carbonyl, nitrile or sulfone group (WO 92/16221).

The activated linkers can be monofunctional, bifunctional, or multifunctional. Useful reagents having a reactive sulfone group that can be used in the methods include, without limitation, chlorosulfone, vinylsulfone and divinylsulfone.

In a specific embodiment, the water soluble polymer is activated with a Michael acceptor. WO 95/13312 describes, inter alia, water soluble sulfone-activated PEGs which are highly selective for coupling with thiol moieties instead of amino moieties on molecules and on surfaces. These PEG derivatives are stable against hydrolysis for extended periods in aqueous environments at pHs of about 11 or less, and can form linkages with molecules to form conjugates which are also hydrolytically stable. The linkage by which the PEGs and the biologically active molecule are coupled includes a sulfone moiety coupled to a thiol moiety and has the structure PEG-SO_2—CH_2—CH_2-S—W, where W represents the biologically active molecule, and wherein the sulfone moiety is vinyl sulfone or an active ethyl sulfone. Two particularly useful homobifunctional derivatives are PEG-bis-chlorosulfone and PEG-bis-vinylsulfone.

U.S. patent application Ser. No. 08/473,809, filed Jun. 7, 1995, the disclosure of which is hereby incorporated by reference, teaches methods of making sulfone-activated linkers by obtaining a compound having a reactive hydroxyl group and converting the hydroxyl group to a reactive Michael acceptor to form an activated linker, with the use of tetrahydrofuran (THF) as the solvent for the conversion. U.S. patent application Ser. No. 08/611,918, filed Mar. 6, 1996, the disclosure of which is hereby incorporated by reference, teaches a process for purifying the activated linkers utilizes hydrophobic interaction chromatography to separate the linkers based on size and end-group functionality.

Polynucleotides

The present invention further provides polynucleotides which encode IL-1ra and variants of IL-1ra. Based upon the present description and using the universal codon table, one of ordinary skill in the art can readily determine all of the nucleic acid sequences which encode the amino acid sequences of IL-1ra and variants of IL-1ra.

Recombinant expression techniques conducted in accordance with the descriptions set forth below may be followed to produce these polynucleotides and to express the encoded proteins. For example, by inserting a nucleic acid sequence which encodes IL-1ra or a variant of IL-1ra into an appropriate vector, one skilled in the art can readily produce large quantities of the desired nucleotide sequence. The sequences can then be used to generate detection probes or amplification primers. Alternatively, a polynucleotide encoding IL-1ra or a variant of IL-1ra can be inserted into an expression vector. By introducing the expression vector into an appropriate host, the desired protein may be produced in large amounts.

As further described herein, there are numerous host/vector systems available for the propagation of desired nucleic acid sequences and/or the production of the desired proteins. These include but are not limited to plasmid, viral and insertional vectors, and prokaryotic and eukaryotic hosts. One skilled in the art can adapt a host/vector system which is capable of propagating or expressing heterologous DNA to produce or express the sequences of the present invention.

Furthermore, it will be appreciated by those skilled in the art that, in view of the present disclosure, the nucleic acid sequences include degenerate nucleic acid sequences encoding IL-1ra having the sequences set forth in FIG. **5** and those nucleic acid sequences which hybridize (preferably under stringent hybridization conditions) to complements of these nucleic acid sequences [Maniatis et al. (1982), *Molecular*

Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, pages 387 to 389]. Exemplary stringent hybridization conditions are hybridization in 4×SSC at 62–67° C., followed by washing in 0.1×SSC at 62–67° C. for approximately an hour. Alternatively, exemplary stringent hybridization conditions are hybridization in 45–55% formamide, 4×SSC at 40–45° C. Also included are DNA sequences which hybridize to the complement of the nucleic acid sequence set forth in SEQ ID NO:1 under relaxed hybridization conditions and which encode the variants of IL-1ra. Examples of such relaxed stringency hybridization conditions are 4×SSC at 45–55° C. or hybridization with 30–40% formamide at 40–45° C.

Also provided by the present invention are recombinant DNA constructs involving vector DNA together with the DNA sequences encoding the desired proteins. In each such DNA construct, the nucleic acid sequence encoding a desired protein (with or without signal peptides) is in operative association with a suitable expression control or regulatory sequence capable of directing the replication and/or expression of the desired protein in a selected host.

Recombinant Expression

Preparation of Polynucleotides

Nucleic acid sequences encoding IL-1ra or variants of IL-1ra can readily be obtained in a variety of ways including, without limitation, chemical synthesis, cDNA or genomic library screening, expression library screening and/or PCR amplification of cDNA. These methods and others which are useful for isolating such nucleic acid sequences are set forth in Sambrook et al. (1989), *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; by Ausubel et al. (1994), eds, *Current Protocols in Molecular Biology,* Current Protocols Press; and by Berger and Kimmel (1987), *Methods in Enzymology: Guide to Molecular Cloning Techniques*, Vol. 152, Academic Press, Inc., San Diego, Calif., the disclosures of which are hereby incorporated by reference.

Chemical synthesis of nucleic acid sequences can be accomplished using methods well known in the art, such as those set forth by Engels et al. (1989), *Angew. Chem. Intl. Ed.,* 28:716–734 and Wells et al. (1985), *Gene,* 34:315, the disclosures of which are hereby incorporated by reference. These methods include, inter alia, the phosphotriester, phosphoramidite and H-phosphonate methods of nucleic acid sequence synthesis. Large nucleic acid sequences, for example those larger than about 100 nucleotides in length, can be synthesized as several fragments. The fragments can then be ligated together to form nucleic acid sequences encoding a desired protein. A preferred method is polymer-supported synthesis using standard phosphoramidite chemistry.

Alternatively, a suitable nucleic acid sequence may be obtained by screening an appropriate cDNA library (i.e., a library prepared from one or more tissue sources believed to express the protein) or a genomic library (a library prepared from total genomic DNA). The source of the cDNA library is typically a tissue from any species that is believed to express a desired protein in reasonable quantities. The source of the genomic library may be any tissue or tissues from any mammalian or other species believed to harbor a gene encoding a desired protein.

Hybridization mediums can be screened for the presence of DNA encoding a desired protein using one or more nucleic acid probes (oligonucleotides, cDNA or genomic DNA fragments that possess an acceptable level of homology to the cDNA or gene to be cloned) that will hybridize selectively with cDNA(s) or gene(s) present in the library. The probes typically used for such screening encode a small region of DNA sequence from the same or a similar species as the species from which the library was prepared. Alternatively, the probes may be degenerate, as discussed herein.

Hybridization is typically accomplished by annealing an oligonucleotide probe or cDNA to the clones under conditions of stringency that prevent non-specific binding but permit binding of those clones that have a significant level of homology with the probe or primer. Typical hybridization and washing stringency conditions depend in part on the size (e.g., number of nucleotides in length) of the cDNA or oligonucleotide probe and whether the probe is degenerate. The probability of identifying a clone is also considered in designing the hybridization medium (e.g., whether a CDNA or genomic library is being screened).

Where a DNA fragment (such as a CDNA) is used as a probe, typical hybridization conditions include those as set forth in Ausubel et al. (1994), eds., supra. After hybridization, the hybridization medium is washed at a suitable stringency depending on several factors such as probe size, expected homology of probe to clone, the hybridization medium being screened, the number of clones being screened and the like. Examples of stringent washing solutions, which are usually low in ionic strength and are used at relatively high temperatures, are as follows: one such stringent wash is 0.015 M NaCl, 0.005 M NaCitrate and 0.1% SDS at 55–65° C.; another such stringent wash is 1 mM Na_2EDTA, 40 mM $NaHPO_4$, pH 7.2 and 1% SDS at about 40–50° C.; and one other stringent wash is 0.2×SSC and 0.1% SDS at about 50–65° C.

There are also exemplary protocols for stringent washing conditions where oligonucleotide probes are used to screen hybridization media. For example, a first protocol uses 6×SSC with 0.05 percent sodium pyrophosphate at a temperature of between about 35° C. and 63° C., depending on the length of the probe. For example, 14 base probes are washed at 35–40° C., 17 base probes at 45–50° C., 20 base probes at 52–57° C., and 23 base probes at 57–63° C. The temperature can be increased 2–3° C. where background non-specific binding appears high. A second protocol uses tetramethylammonium chloride (TMAC) for washing. One such stringent washing solution is 3 M TMAC, 50 mM Tris-HCl, pH 8.0 and 0.2% SDS.

Another suitable method for obtaining a suitable nucleic acid sequence is the polymerase chain reaction (PCR). In this method, cDNA is prepared from poly(A)+RNA or total RNA using the enzyme reverse transcriptase. Two primers, typically complementary to two separate regions of CDNA (oligonucleotides) encoding the desired protein, are then added to the cDNA along with a polymerase such as Taq polymerase, and the polymerase amplifies the cDNA region between the two primers.

The oligonucleotide sequences selected as probes or primers should be of adequate length and sufficiently unambiguous as to minimize the amount of non-specific binding that may occur during screening or PCR amplification. The

actual sequence of the probes or primers is usually based on conserved or highly homologous sequences or regions. Optionally, the probes or primers can be fully or partially degenerate, i.e., can contain a mixture of probes/primers, all encoding the same amino acid sequence but using different codons to do so. An alternative to preparing degenerate probes is to place an inosine in some or all of those codon positions that vary by species. The oligonucleotide probes or primers may be prepared by chemical synthesis methods for DNA, as described above.

Vectors

DNA encoding the desired proteins may be inserted into vectors for further cloning (amplification of the DNA) or for expression. Suitable vectors are commercially available, or the vector may be specifically constructed. The selection or construction of an appropriate vector will depend on (1) whether it is to be used for DNA amplification or for DNA expression, (2) the size of the DNA to be inserted into the vector and (3) the intended host cell to be transformed with the vector.

The vectors each involve a nucleic acid sequence which encodes a desired protein operatively linked to one or more of the following expression control or regulatory sequences capable of directing, controlling or otherwise effecting the expression of a desired protein by a selected host cell. Each vector contains various components, depending on its function (amplification of DNA or expression of DNA) and its compatibility with the intended host cell. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more selection or marker genes, a promoter, an enhancer element, a transcription termination sequence and the like. These components may be obtained from natural sources or be synthesized by known procedures.

Examples of suitable prokaryotic cloning vectors include bacteriophages such as lambda derivatives, or plasmids from *E. coli* (e.g. pBR322, col E1, pUC, the F-factor and Bluescript® plasmid derivatives (Stratagene, LaJolla, Calif.)). Other appropriate expression vectors, of which numerous types are known in the art for the host cells described below, can also be used for this purpose.

Signal Sequence

The nucleic acid encoding a signal sequence may be inserted 5' of the sequence encoding a desired protein, e.g, it may be a component of a vector or it may be a part of a nucleic acid encoding the desired protein. For example, the nucleic acid encoding the native signal sequence of IL-1ra is known (U.S. Pat. No. 5,075,222).

Origin of Replication

Expression and cloning vectors each generally include a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. In a cloning vector, this sequence is typically one that enables the vector to replicate independently of the host chromosomal DNA and includes an origin of replication or autonomously replicating sequence. Such sequences are well known. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, and various origins (e.g., SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication is not needed for mammalian expression vectors (for example, the SV40 origin is often used only because it contains the early promoter).

Selection Gene

The expression and cloning vectors each typically contain a selection gene. This gene encodes a "marker" protein necessary for the survival or growth of the transformed host cells when grown in a selective culture medium. Host cells that are not transformed with the vector will not contain the selection gene and, therefore, they will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate or tetracycline; (b) complement auxotrophic deficiencies or (c) supply critical nutrients not available from the culture medium.

Other selection genes may be used to amplify the genes to be expressed. Amplification is the process wherein genes which are in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples of suitable selectable markers for mammalian cells include dihydrofolate reductase (DHFR) and thymidine kinase. The cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of the markers present in the vectors. Selection pressure is imposed by culturing the transformed cells under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes a desired protein. As a result, increased quantities of a desired protein are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate, a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is used is the Chinese hamster ovary cell line deficient in DHFR activity (Urlaub and Chasin (1980), *Proc. Natl. Acad. Sci., USA,* 77(7):4216–4220, the disclosure of which is hereby incorporated by reference). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene and, concomitantly, multiple copies of other DNA present in the expression vector, such as the DNA encoding a desired protein.

Promoter

Expression and cloning vectors each will typically contain a promoter that is recognized by the host organism and is operably linked to a nucleic acid sequence encoding a desired protein. A promoter is an untranslated sequence located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that controls the transcription and translation of a particular nucleic acid sequence, such as that encoding a desired protein. A promoter may be conventionally grouped into one of two classes, inducible promoters or constitutive promoters. An inducible promoter initiates increased levels of transcription from DNA under its control in response to some change in culture conditions, such as the presence or absence of a nutrient or a change in temperature. A large number of promoters, recognized by a variety of potential host cells, are well known. A promoter may be operably linked to the DNA encoding a desired protein by removing the promoter from the source DNA by restriction enzyme digestion and inserting the desired promoter sequence. The native IL-1ra

promoter sequence may be used to direct amplification and/or expression of the DNA encoding a desired protein. A heterologous promoter is preferred, however, if it permits greater transcription and higher yields of the expressed protein as compared to the native promoter and if it is compatible with the host cell system that has been selected for use. For example, any one of the native promoter sequences of other IL-1 family members may be used to direct amplification and/or expression of the DNA encoding a desired protein.

Promoters suitable for use with prokaryotic hosts include the beta-lactamase and lactose promoter systems; alkaline phosphatase, a tryptophan (trp) promoter system; a bacterial luminescence (luxR) gene system and hybrid promoters such as the tac promoter. Other known bacterial promoters are also suitable. Their nucleotide sequences have been published, thereby enabling one skilled in the art to ligate them to the desired DNA sequence(s) using linkers or adaptors as needed to supply any required restriction sites.

Suitable promoting sequences for use with yeast hosts are also well known in the art. Suitable promoters for use with mammalian host cells are well known and include those obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and, most preferably, Simian Virus 40 (SV40). Other suitable mammalian promoters include heterologous mammalian promoters, e.g., heat-shock promoters and the actin promoter.

Enhancer Element

The expression and cloning vectors each will typically contain an enhancer sequence to increase the transcription by higher eukaryotes of a DNA sequence encoding a desired protein. Enhancers are cis-acting elements of DNA, usually from about 10–300 bp in length, that act on the promoter to increase its transcription. Enhancers are relatively orientation and position independent. They have been found 5' and 3' to the transcription unit. Yeast enhancers are advantageously used with yeast promoters. Several enhancer sequences available from mammalian genes are known (e.g., globin, elastase, albumin, alpha-feto-protein and insulin). Additionally, viral enhancers such as the SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer, and adenovirus enhancers are exemplary enhancing elements for the activation of eukaryotic promoters. While an enhancer may be spliced into a vector at a position 5' or 3' to a DNA encoding a desired protein, it is typically located at a site 5' from the promoter.

Transcription Termination

Expression vectors used in eukaryotic host cells each will typically contain a sequence necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and occasionally 3' untranslated regions of eukaryotic DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding a desired protein.

Vector Construction

The construction of suitable vectors, each containing one or more of the above-listed components (together with the coding sequence encoding a desired protein) may be accomplished by standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored and religated in the desired order to generate the vector required. To confirm that the correct sequence has been constructed, the ligation mixture may be used to transform *E. coli,* and successful transformants may be selected by known techniques as described above. Quantities of the vector from the transformants are then prepared, analyzed by restriction endonuclease digestion and/or sequenced to confirm the presence of the desired construct.

A vector that provides for the transient expression of DNA encoding a desired protein in mammalian cells may also be used. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of the desired protein encoded by the expression vector. Each transient expression system, comprising a suitable expression vector and a host cell, allows for the convenient positive identification of proteins encoded by cloned DNAs as well as for the rapid screening of such proteins for desired biological or physiological properties, i.e., identifying a biologically-active variant of IL-1ra protein.

Host Cells

Any of a variety of recombinant host cells, each of which contains a nucleic acid sequence for use in expressing a desired protein, is also provided by the present invention. Exemplary prokaryotic and eukaryotic host cells include bacterial, mammalian, fungal, insect, yeast or plant cells.

Prokaryotic host cells include but are not limited to eubacteria such as Gram-negative or Gram-positive organisms (e.g., *E. coli* (HB101, DH5a, DH10 and MC1061); Bacilli, such as *B. subtilis;* Pseudomonas, such as *P. aeruginosa;* Streptomyces spp.; *Salmonella typhimurium;* or *Serratia marcescans.* As a specific embodiment, a desired protein may be expressed in *E. coli.*

In addition to prokaryotic host cells, eukaryotic microbes such as filamentous fungi or yeast may be suitable hosts for the expression of a desired protein. *Saccharomyces cerevisiae,* or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms, but a number of other genera, species and strains are well known and commonly available.

A desired protein may be expressed in glycosylated form by any one of a number of suitable host cells derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture might be used, whether such culture involves vertebrate or invertebrate cells, including plant and insect cells. As a specific embodiment, a desired protein may be expressed in baculovirus cells.

Vertebrate cells may be used, as the propagation of vertebrate cells in culture (tissue culture) is a well-known procedure. Examples of useful mammalian host cell lines include but are not limited to monkey kidney CV1 line transformed by SV40 (COS-7), human embryonic kidney line (293 cells or 293 cells subcloned for growth in suspension culture), baby hamster kidney cells and Chinese hamster ovary cells. Other suitable mammalian cell lines include but are not limited to HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, and BHK or HaK

hamster cell lines. As a specific embodiment, a desired protein may be expressed in COS cells.

A host cell may be transfected and preferably transformed with a desired nucleic acid under appropriate conditions permitting the expression of the nucleic acid sequence. The selection of suitable host cells and methods for transformation, culture, amplification, screening and product production and purification are well known in the art (Gething and Sambrook (1981), *Nature,* 293:620–625 or, alternatively, Kaufman et al. (1985), *Mol. Cell. Biol.,* 5(7) :1750–1759, or U.S. Pat. No. 4,419,446, the disclosures of which are hereby incorporated by reference). For example, for mammalian cells without cell walls, the calcium phosphate precipitation method may be used. Electroporation, micro-injection and other known techniques may also be used.

It is also possible that a desired protein may be produced by homologous recombination or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the desired protein. Homologous recombination is a technique originally developed for targeting genes to induce or correct mutations in transcriptionally-active genes (Kucherlapati (1989), *Prog. in Nucl. Acid Res. and Mol. Biol.,* 36:301, the disclosure of which is hereby incorporated by reference). The basic technique was developed as a method for introducing specific mutations into specific regions of the mammalian genome (Thomas et al. (1986), *Cell,* 44:419–428; Thomas and Capecchi (1987), *Cell,* 51:503–512 and Doetschman et al. (1988), *Proc. Natl. Acad. Sci.,* 85:8583–8587, the disclosures of which are hereby incorporated by reference) or to correct specific mutations within defective genes (Doetschman et al. (1987), *Nature,* 330:576–578, the disclosure of which is hereby incorporated by reference). Exemplary techniques are described in U.S. Pat. No. 5,272, 071; WO 92/01069; WO 93/03183; WO 94/12650 and WO 94/31560, the disclosures of which are hereby incorporated by reference.

Through homologous recombination, the DNA sequence to be inserted into the genome can be directed to a specific region of the gene of interest by attaching it to targeting DNA. The targeting DNA is DNA that is complementary (homologous) to a region of the genomic DNA. Small pieces of targeting DNA that are complementary to a specific region of the genome are put in contact with the parental strand during the DNA replication process. A general property of DNA that has been inserted into a cell is to hybridize and therefore recombine with other pieces of endogenous DNA through shared homologous regions. If this complementary strand is attached to an oligonucleotide that contains a mutation or a different sequence of DNA, it too is incorporated into the newly synthesized strand as a result of the recombination. As a result of the proofreading function, it is possible for the new sequence of DNA to serve as the template. Thus, the transferred DNA is incorporated into the genome.

If the sequence of a particular gene is known, such as the nucleic acid sequence of a desired protein, the expression control sequence (a piece of DNA that is complementary to a selected region of the gene) can be synthesized or otherwise obtained, such as by appropriate restriction of the native DNA at specific recognition sites bounding the region of interest. This piece serves as a targeting sequence upon insertion into the cell and will hybridize to its homologous region within the genome. If this hybridization occurs during DNA replication, this piece of DNA, and any additional sequence attached thereto, will act as an Okazaki fragment and will be backstitched into the newly synthesized daughter strand of DNA.

Attached to these pieces of targeting DNA are regions of DNA which may interact with the expression of a desired protein. For example, a promoter/enhancer element, a suppressor or an exogenous transcription modulatory element is inserted into the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired protein. The control element does not encode a desired protein but instead controls a portion of the DNA present in the host cell genome. Thus, the expression of a desired protein may be achieved not by transfection of DNA that encodes a desired protein, but rather by the use of targeting DNA (containing regions of homology with the endogenous gene of interest), coupled with DNA regulatory segments that provide the endogenous gene sequence with recognizable signals for transcription of a desired protein.

Culturing the Host Cells

The method for culturing each of the one or more recombinant host cells for production of a desired protein will vary depending upon many factors and considerations; the optimum production procedure for a given situation will be apparent to those skilled in the art through minimal experimentation. Such recombinant host cells are cultured in a suitable medium and the expressed protein is then optionally recovered, isolated and purified from the culture medium (or from the cell, if expressed intracellularly) by an appropriate means known to those skilled in the art.

Specifically, each of the recombinant cells used to produce a desired protein may be cultured in media suitable for inducing promoters, selecting suitable recombinant host cells or amplifying the gene encoding the desired protein. The media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as gentamicin), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or another energy source. Other supplements may also be included at appropriate concentrations, as will be appreciated by those skilled in the art. Suitable culture conditions, such as temperature, pH and the like, are also well known to those skilled in the art for use with the selected host cells.

The resulting expression product may then be purified to near homogeneity by using procedures known in the art. Exemplary purification techniques are taught in U.S. Pat. No. 5,075,222, and WO 91/08285. Preferably, expression product is produced in a substantially pure form. By "substantially pure" is meant IL-1ra, in an unmodified form, has a comparatively high specific activity, preferably in the range of approximately 150,000–500,000 receptor units/mg as defined in Hannum et al. (1990), *Nature,* 343:336–340 and Eisenberg et al. (1990), *Nature,* 343:341–346, both of

which are specifically incorporated herein by reference. It is to be recognized, however, that a variant of IL-1ra can have a different specific activity.

Pharmaceutical Compositions

Pharmaceutical compositions generally will each typically include a therapeutically effective amount of at least one of an IL-1ra, a variant of IL-1ra or a chemical derivative thereof (collectively hereinafter referred to as an "IL-1ra product") in a vehicle. In one embodiment, the vehicle includes one or more pharmaceutically and physiologically acceptable formulation materials. In one embodiment, the IL-1ra product is formulated in a vehicle which does not contain a controlled release material. In another embodiment, the IL-1ra product is formulated in a vehicle which does contain a controlled release material.

The primary solvent in a vehicle may be either aqueous or non-aqueous in nature. In addition, the vehicle may contain other pharmaceutically acceptable excipients for modifying or maintaining the pH, preferably between 6.0 and 7.0, more preferably 6.5 (e.g., buffers such as citrates, phosphates and amino acids such glycine); bulking agents for lyophilized formulation (e.g., mannitol and glycine); osmolarity (e.g., mannitol and sodium chloride); surfactants (e.g., polysorbate 20, polysorbate 80, triton, and pluronics); viscosity; clarity; color; sterility; stability (e.g., sucrose and sorbitol); antioxidants (e.g., sodium sulfite and sodium hydrogensulfite); preservatives (e.g., benzoic acid and salicylic acid); odor of the formulation; flavoring and diluting agents; rate of dissolution (e.g., solubilizers or solubilizing agents such as alcohols, polyethylene glycols and sodium chloride); rate of release; emulsifying agents; suspending agents; solvents; fillers; delivery vehicles; diluents; excipients and/or pharmaceutical adjuvants. Other effective administration forms such as parenteral inhalant mists, orally-active formulations or suppositories are also envisioned. The optimal pharmaceutical formulation for a desired protein will be determined by one skilled in the art depending upon the route of administration and desired dosage (*Remington's Pharmaceutical Sciences,* 18th Ed. (1990), Mack Publishing Co., Easton, Pa. 18042, pages 1435–1712, the disclosure of which is hereby incorporated by reference). Specific pharmaceutical formulations are as follows: 10 millimolar sodium citrate, 140 millimolar sodium chloride, 0.5 millimolar EDTA, 0.1% polysorbate 80 (w/w) in water, pH 6.5 ("citrate buffer formulation"); and 10 millimolar sodium phosphate, 140 millimolar sodium chloride, between 0.1% (wt/wt) and 0.01% polysorbate 80 (w/w) in water, and, optionally, 0.5 millimolar EDTA, pH 6.5 ("phosphate buffer formulation").

In another embodiment the controlled release polymer may be selected from bulk erosion polymers (e.g., poly (lactic-co-glycolic acid) (PLGA) copolymers, PLGA polymer blends, block copolymers of PEG, and lactic and glycolic acid, poly(cyanoacrylates)); surface erosion polymers (e.g., poly(anhydrides) and poly(ortho esters)); hydrogel esters (e.g., pluronic polyols, poly(vinyl alcohol), poly (vinylpyrrolidone), maleic anhydride-alkyl vinyl ether copolymers, poly(2-hydroxyethyl methacrylate) (pHEMA), methacrylic acid (MAA), blends of pHEMA and MAA, cellulose (e.g., carboxymethylcellulose), hyaluronan, alginate, collagen, gelatin, albumin, and starches and dextrans) and composition systems thereof; or preparations of liposomes or microspheres. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives. The optimal pharmaceutical formulation for a desired protein will be determined by one skilled in the art depending upon the route of administration and desired dosage. Exemplary pharmaceutical compositions are disclosed in Gombotz and Pettit (1995), *Bioconjugate Chem.,* 6:332–351 and *Remington's Pharmaceutical Sciences,* 18th Ed. (1990), Mack Publishing Co., Easton, Pa. 18042, pages 1435–1712, the disclosures of which are hereby incorporated by reference. Specific controlled release compositions are available from the following suppliers: DepoTech Corp., San Diego, Calif. (Depofoam™, a multivesicular liposome) and Alkermes, Inc., Cambridge, Mass. (ProLease™, a PLGA microsphere).

In a specific embodiment, the present invention is directed to drug delivery systems based on hyaluronan in soluble or non-soluble cross-linked forms. As used herein, hyaluronan is intended to include hyaluronan, hyaluronic acid, salts thereof (such as sodium hyaluronate), esters, ethers, enzymatic derivatives and cross-linked gels of hyaluronic acid, and chemically modified derivatives of hyaluronic acid (such as hylan). Non-modified or modified hyaluronic acid serves as a vehicle which provides slow release of a drug from a system.

The hyaluronan may be of any type already recognized as useful for such purposes. It may be extracted from various non-limiting materials such as rooster combs or umbilical cords or from bacterial cultures such as those of hemolytic group A or C streptococci. Exemplary forms of hyaluronan are disclosed in Peyron and Balazs (1974), *Path. Biol.,* 22(8):731–736; Isdale et al. (1991), *J. Drug Dev.,* 4(2) :93–99; Larsen et al. (1993), *Journal of Biomedical Materials Research,* 27:1129–1134; Namiki, et al. (1982), *International Journal of Clinical Pharmacology, Therapy and Toxicology,* 20(11):501–507; Meyer et al. (1995), *Journal of Controlled Release,* 35:67–72; Kikuchi et al. (1996), *Osteoarthritis and Cartilage,* 4:99–110; Sakakibara et al. (1994), *Clinical Orthopaedics and Related Research,* 299:282–292; Meyers and Brandt (1995), 22(9):1732–1739; Laurent et al. (1995), *Acta Orthop Scand,* 66(266):116–120; Cascone et al. (1995), *Biomaterials,* 16(7):569–574; Yerashalmi et al. (1994), *Archives of Biochemistry and Biophysics,* 313(2):267–273; Bernatchez et al. (1993), *Journal of Biomedical Materials Research,* 27(5):677–681; Tan et al. (1990), *Australian Journal of Biotechnology,* 4(1) :38–43; Gombotz and Pettit (1995), *Bioconjugate Chem.,* 6:332–351; U.S. Pat. Nos. 4,582,865, 4,605,691, 4,636,524, 4,713,448, 4,716,154, 4,716,224, 4,772,419, 4,851,521, 4,957,774, 4,863,907, 5,128,326, 5,202,431, 5,336,767, 5,356,883; European Patent Application Nos. 0 507 604 A2 and 0 718 312 A2; and WO 96/05845, the disclosures of which are hereby incorporated by reference.

The hyaluronan should be pure enough to avoid provoking an adverse or toxic reaction in the mammal being treated. This implies that it be free of pyrogens and have a sufficiently low level of proteins and/or nucleic acids with which hyaluronan is naturally associated, so that no substantial immune reaction is provoked. Suitable purification procedures are described in U.S. Pat. Nos. 4,141,973, 5,411,

874, 5,442,053, 5,559,104, 5,563,051 and Japanese Patent Application Nos. 14594/1977, 67100/1979 and 74796/1980, the disclosures of which are hereby incorporated by reference.

The hyaluronan may be in its free acid form or in any pharmacologically acceptable salt form. Also, as salts, there may be mentioned an alkali metal salt such as sodium or potassium salt and an alkaline earth metal salt such as calcium or magnesium salt. The preferred source of hyaluronan is a culture of an appropriate microorganism.

Hyaluronan having a molecular weight within a wide range can be used in the present invention. The molecular weight of hyaluronan is generally between 0.1×10^6 and 1×10^7, preferably between 0.5×10^6 and 5×10^6, more preferably between 1×10^6 and 5×10^6 and most preferably between 1×10^6 and 4×10^6 (e.g., between 1×10^6 and 2×10^6).

Increasing the molecular weight of hyaluronan by crosslinking has been accomplished in a number of ways. Sakuria et al. in U.S. Pat. No. 4,716,224, disclose crosslinked hyaluronic acid or salts thereof prepared by crosslinking hyaluronic acid or its salts with a polyfunctional epoxide. In U.S. Pat. No. 4,863,907, Sakuri et al. disclose crosslinked glycosaminoglycan or salts thereof prepared by crosslinking a glycosaminoglycan or a salt thereof with a polyfunctional epoxy compound. Huang et al., in European Patent Application No. 0 507 604 A2, disclose ionically crosslinked carboxyl-containing polysaccharides where the crosslinking agent is a compound possessing a trivalent cation. Malson et al., in U.S. Pat. Nos. 4,716,154 and 4,772,419 disclose crosslinking hyaluronic acid with bi- or polyfunctional epoxides or their corresponding halohydrins, epihalohydrins or halides, and divinyl sulfone. In. U.S. Pat. No. 4,957,744, della Valle et al. disclose crosslinking esters of hyaluronic acid prepared by esterifying the carboxyl groups of hyaluronic acid with polyhydric alcohols. Balazs et al., in U.S. Pat. Nos. 4,582,865, 4,605, 691 and 4,636,524, disclose crosslinking of hyaluronic acid and its salts, and of other polysaccharides, by reaction with divinyl sulfone. In U.S. Pat. Nos. 5,128,326 and 4,582,865, Balazs et al. disclose crosslinking hyaluronic acid with formaldehyde, epoxides, polyaziridyl compounds and divinyl sulfone. In U.S. Pat. No. 4,713,448, Balazs et al. disclose chemically modifying hyaluronic acid by reaction with aldehydes such as formaldehyde, glutaraldehyde and glyoxal and teach the possibility that crosslinking has occurred. In U.S. Pat. No. 5,356,883, Kuo et al. disclose crosslinking hyaluronic acid by reaction with biscarbodiimides. In EP 0 718 312 A2, Nguyen discloses crosslinking hyaluronic acid or its salts, and of other polysaccharides, by reaction with di- or polyanhydrides.

The hyaluronan concentration in the products, based on the soluble polymers, can be in the range of from about 0.05% to 5% by wt. and higher, depending on the end use of the product, preferably between 0.1% to 4% by wt, more preferably between 1% to 3% by weight. The concentration of IL-1 inhibitor can be varied over very broad limits and preferably should be chosen depending upon the solubility of the IL-1 inhibitor, its pharmacological activity, the desirable effect of the end product, etc.

The crosslinked hyaluronan is usually dissolved in a solvent (e.g., physiological saline) to such a sufficient viscosity to pass through an injection needle. Low viscosity material greatly facilitates the injection by allowing, for instance, the use of a concentrated aqueous hyaluronan solution in practical size doses. Thus, for example, a 1% aqueous solution of hyaluronan can be readily utilized for injection doses of about 10 milliliters, which each contain about 100 milligrams of active ingredient if its viscosity is less than about 200 c/s at 37° C. (as determined using a Cannon-Manning Semi-Micro Viscometer according to the procedures in ASTM D 445 and D 2515).

The drug delivery system according to the present invention includes the following:

1) hyaluronan solutions in which a drug substance is dissolved or dispersed;
2) a cross-linked hyaluronan gel forming a macromolecular "cage" in which a drug substance is dispersed;
3) A cross-linked mixed gel of hyaluronan and at least one other hydrophilic polymer in which a drug substance is dispersed; and
4) A cross-linked gel of hyaluronan or cross-linked mixed gel of hyaluronan and at least one other hydrophilic polymer containing a drug substance which is covalently attached to the macromolecules of hyaluronic acid or the other polymer.

There are several methods for combining a drug with the gel and, accordingly, several types of products which can be obtained.

One of the methods comprises diffusing a drug into a gel when the gel is put into a solution of the drug. The diffusion process is usually slow and depends upon the drug concentration, temperature of the solution, size of the gel particles, etc. The product obtained by this method is a gel in which a drug substance is uniformly dispersed.

The same type of product can be obtained by dehydrating a hyaluronan gel and reswelling it in a drug solution. To dehydrate a gel one can use a water-miscible organic solvent or, alternatively, water from a gel can be removed by drying. However, it is preferable to use a solvent because after drying at a low or elevated temperature, the gel cannot re-swell to its initial degree of swelling. On the other hand, after dehydrating with a solvent, the gel swells to the same volume it had before the treatment. Preferable solvents are ethanol and isopropanol, and ketones such as acetone, though other solvents can also be used.

Yet another method can be used to obtain products of this type. This method comprises allowing a concentrated hyaluronic acid gel resulting from a cross-linking reaction previously carried out in a relatively concentrated solution of hyaluronan to swell in a solution of a drug substance.

Although these three methods all result in products which are essentially the same, each of the methods has certain advantages when compared to any of the other methods for any specific product and, hence, the choice of method should be made with consideration given to such parameters as nature of the drug, the desired concentration of the drug in the system, the delivery rate, etc.

In order to obtain a hyaluronan solution in which a drug substance is dissolved or dispersed, any conventional method can be used. Hyaluronan from any source can be dissolved in water or in physiological saline to a desired concentration and then a drug is dissolved or dispersed in the resulting solution. Alternatively, a solution or dispersion of a drug can be mixed with hyaluronan solution. The polymer

concentration is chosen depending upon the end use of the product and the molecular weight of hyaluronan. The drug concentration is chosen depending upon the desired activity of the product.

To load a cross-linked swollen gel with a drug using the diffusion process, the gel can be put into a drug solution. The time for completion of this process depends upon gel particle size, gel swelling ratio, temperature of the process, stirring, concentration of the drug in the solution, etc. By proper combination of these parameters, a swollen gel can be loaded with a drug in a relatively short period of time.

To dehydrate a cross-linked gel with a solvent, it is enough to put the gel in any form (i.e., as fine particles or as a membrane) into a solvent, preferably a volatile solvent (e.g., isopropanol), and keep it in the solvent for a sufficient amount of time to remove water from the gel. The degree of water removal depends upon the size of the particles or the membrane thickness, the gel/solvent ratio, etc. Treatment with a solvent can be repeated several times, if desired. The solvent from the gel can be removed by drying under normal pressure or in a vacuum at room or elevated temperature. The thusly dehydrated gel, when put into a drug solution, reswells to the initial swelling ratio.

Specific hyaluronan compositions are available from the following suppliers: BioMatrix, Inc. Ridgefield, N.J. (Synvisc™, a 90:10 mixture of a hylan fluid and hylan gel); Fidia S.p.A., Abano Terme, Italy (Hyalgan™, the sodium salt of a rooster comb-derived hyaluronic acid (~500,000 to ~700,000 MW)); Kaken Pharmaceutical Co., Ltd., Tokyo, Japan (Artz™, a 1% solution of a rooster-comb derived hyaluronic acid, ~700,000 MW); Pharmacia AB, Stockholm, Sweden (Healom™, a rooster-comb derived hyaluronic acid, ~4×10^6 MW); Genzyme Corporation, Cambridge, Mass. (Surgicoat™, a recombinant hyaluronic acid); Pronova Biopolymer, Inc. Portsmouth, N.H. (Hyaluronic Acid FCH, a high molecular weight (e.g., ~$1.5–2.2\times10^6$ MW) hyaluronic acid prepared from cultures of *Streptococcus zooepidemicus*; Sodium Hyaluronate MV, ~$1.0–1.6\times10^6$ MW and Sodium Hyaluronate LV, ~$1.5–2.2\times10^6$ MW); Calbiochem-Novabiochem AB, Lautelfingen, Switzerland (Hyaluronic Acid, sodium salt (1997 company catalog number 385908) prepared from Streptococcus sp.); Intergen Company, Purchase, N.Y. (a rooster-comb derived hyaluronic acid, >1×10^6 MW); Diosynth Inc., Chicago, Ill.; Amerchol Corp., Edison, N.J. and Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan.

In a preferred embodiment of the present invention, IL-1ra in the form of finely divided particles is dissolved or suspended in a 0.1–5% w/v solution of hyaluronan or its salt (e.g., sodium hyaluronate) as a dry powder or in water or an aqueous solvent (e.g., physiological saline solutions such as a water-soluble sodium salt, 3 to 5% glucose solutions and 3 to 5% xylitol solutions and citrate or phosphate buffer formulations). The hyaluronan and IL-1ra can be mixed using means such as injecting IL-1ra solution back and forth from one syringe to a second syringe containing the hyaluronan, or by stirring, or by microfluidization. The IL-1ra mixtures can be stored at 0–5° C. without degradation or aggregation of the protein. The hyaluronan concentration can range from 0.1–5% w/v, but the preferred concentration is 2%. Likewise, the final IL-1ra concentration in the preparation can be from 0.1–200 mg/ml, but the preferred concentration is 100 mg/ml. The resulting solution or suspension is preferably adjusted so that the pH value is from 6.0 to 7.5.

Once the pharmaceutical compositions have been formulated, each may be stored in a sterile vial as a solution, suspension, gel, emulsion, solid, or a dehydrated or lyophilized powder. Such compositions may be stored either in ready-to-use form or in a form (e.g., lyophilized) requiring reconstitution prior to administration. The preferred storage of such formulations is at temperatures at least as low as 4° C. and preferably at −70° C. It is also preferred that such formulations containing IL-1ra are stored and administered at or near physiological pH. It is presently believed that storage and administration in a formulation at a high pH (i.e., greater than 8) or at a low pH (i.e., less than 5) is undesirable, with a pH of preferably between 6.0 and 7.0 being preferable and a pH of 6.5 being more preferable.

In a specific embodiment, the present invention is directed to kits for producing a single-dose administration unit. The kits may each contain both a first container having a dried protein and a second container having an aqueous formulation. Kits included within the scope of this invention are single and multi-chambered pre-filled syringes; exemplary pre-filled syringes (e.g., liquid syringes, and lyosyringes such as Lyo-Ject®, a dual-chamber pre-filled lyosyringe) are available from Vetter GmbH, Ravensburg, Germany.

An IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) may be administered to a patient in a therapeutically effective amount for the treatment of IL-1-mediated diseases, as defined above, including rheumatic diseases (e.g., lyme disease, juvenile (rheumatoid) arthritis, osteoarthritis, psoriatic arthritis, rheumatoid arthritis and staphylococcal-induced ("septic") arthritis). The term "patient" is intended to encompass animals (e.g., cats, dogs and horses) as well as humans.

Further, the IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) each may be administered via topical, enteral or parenteral administration including, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intra-articular, subcapsular, subarachnoid, intraspinal, intraventricular and intrasternal injection and infusion. An IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) may also be administered via oral administration or be administered through mucus membranes, that is, intranasally, sublingually, buccally or rectally for systemic delivery.

It is preferred that an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) is administered via intra-articular, subcutaneous, intramuscular or intravenous injection. Additionally, an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) may be administered by a continuous infusion (e.g., constant or intermittent implanted or external infusion flow-modulating

devices) so as to continuously provide the desired level of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in the blood for the duration of the administration. This is most preferably accomplished by means of continuous infusion via, e.g., mini-pump such as osmotic mini-pump. In these ways, one can be assured that the amount of drug is maintained at the desired level and one can take blood samples and monitor the amount of drug in the bloodstream. Various pumps are commercially available, such as the Alzet osmotic pump, model 2MLI, Alza Corp., Palo Alto, Calif.

By way of example but not limitation, in one specific embodiment IL-1 inhibitors (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) may be administered intra-articularly for the treatment of rheumatoid arthritis and osteoarthritis. By way of example but not limitation in another specific embodiment, IL-1 inhibitors (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) may be administered subcutaneously or intramuscularly for the treatment of rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, multiple myeloma, or myelogenous (e.g., AML and CML) and other leukemias. By way of example but not limitation, in a still further specific embodiment IL-1 inhibitors (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) may be administered intravenously for the treatment of brain injury as a result of trauma, epilepsy, hemorrhage or stroke, or for the treatment of graft-versus-host disease; or administered intraventricularly for the treatment of brain injury as a result of trauma.

Regardless of the manner of administration, the treatment of IL-1-mediated disease requires a dose or total dose regimen of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) of effective amounts, i.e., effective to prevent, reduce or alleviate symptoms of the disease, such as to counteract progressive cartilage destruction of a joint as caused by degradation of proteoglycans which are a molecular component of articular cartilage. As hyaluronan and IL-1ra are naturally occurring substances in mammals, it is believed that there is no inherent upper limit to the tolerable dose. However, as in all medicinal treatments, it is prudent to use no more than is necessary to achieve the desired effect.

The specific dose is calculated according to the approximate body weight or surface area of the patient. Other factors in determining the appropriate dosage can include the disease or condition to be treated or prevented, the severity of the disease, the route of administration, and the age, sex and medical condition of the patient. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those skilled in the art, especially in light of the dosage information and assays disclosed herein. The dosage can also be determined through the use of known assays for determining dosages used in conjunction with appropriate dose-response data.

The frequency of dosing depends on the disease and condition of the patient, as well as the pharmacokinetic parameters of the IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) used in the formulation, and the route of administration. The IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) may be administered once, or in cases of severe and prolonged disorders, administered daily in less frequent doses or administered with an initial bolus dose followed by a continuous dose or sustained delivery. It is also contemplated that other modes of continuous or near-continuous dosing may be practiced.

Preferred modes of using IL-1ra products for treatment of IL-1-mediated diseases, as defined above, including acute and chronic inflammation such as inflammatory conditions of a joint (e.g., rheumatoid arthritis and psoriatic arthritis), are set forth in AU 9173636. These modes include: (1) a single intra-articular injection of IL-1ra given periodically as needed to prevent or remedy the flare-up of arthritis and (2) periodic subcutaneous injections of IL-1ra product. When administered parenterally, the unit dose may be up to 200 mg, generally up to 150 mg and more generally up to 100 mg. When administered into an articular cavity, the pharmaceutical composition is preferably administered as a single injection from a 3 to 10 ml syringe containing a dose up to 200 mg/ml, generally up to 150 mg and more generally up to 100 mg of IL-1 product dissolved in isotonic phosphate buffered saline. The preparation is administered into an articular cavity at a frequency of once every 7 to 10 days. In such a manner, administration is continuously conducted 4 to 5 times while varying the dose if necessary.

Pharmaceutical compositions of the present invention may be administered with other therapeutics suitable for the indication being treated. An IL-1 inhibitor product (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) and any of one or more additional anti-inflammatory drugs may be administered separately or in combination. Information regarding the following compounds can be found in The Merck Manual of Diagnosis and Therapy, Sixteenth Edition, Merck, Sharp & Dohme Research Laboratories, Merck & Co., Rahway, N.J. (1992) and in Pharmaprojects, PJB Publications Ltd.

Present treatment of IL-1-mediated diseases, as defined above, including acute and chronic inflammation such as rheumatic diseases (e.g., lyme disease, juvenile (rheumatoid) arthritis, osteoarthritis, psoriatic arthritis, rheumatoid arthritis and staphylococcal-induced ("septic") arthritis) includes first line drugs for control of pain and inflammation classified as non-steroidal, anti-inflammatory drugs (NSAIDs). Secondary treatments include corticosteroids, slow acting antirheumatic drugs (SAARDs) or disease modifying (DM) drugs.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) and any of one or more NSAIDs for the treatment of IL-1-mediated diseases, as defined above, including acute and chronic inflammation such as rheumatic diseases (e.g., lyme disease, juvenile (rheumatoid) arthritis, osteoarthritis, psoriatic arthritis, rheu-

matoid arthritis and staphylococcal-induced ("septic") arthritis); and graft versus host disease. NSAIDs owe their anti-inflammatory action, at least in part, to the inhibition of prostaglandin synthesis (Goodman and Gilman in "The Pharmacological Basis of Therapeutics," MacMillan 7th Edition (1985)). NSAIDs can be characterized into nine groups: (1) salicylic acid derivatives; (2) propionic acid derivatives; (3) acetic acid derivatives; (4) fenamic acid derivatives; (5) carboxylic acid derivatives; (6) butyric acid derivatives; (7) oxicams; (8) pyrazoles and (9) pyrazolones.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) with any of one or more salicylic acid derivatives, prodrug esters or pharmaceutically acceptable salts thereof. Such salicylic acid derivatives, prodrug esters and pharmaceutically acceptable salts thereof comprise: acetaminosalol, aloxiprin, aspirin, benorylate, bromosaligenin, calcium acetylsalicylate, choline magnesium trisalicylate diflusinal, etersalate, fendosal, gentisic acid, glycol salicylate, imidazole salicylate, lysine acetylsalicylate, mesalamine, morpholine salicylate, 1-naphthyl salicylate, olsalazine, parsalmide, phenyl acetylsalicylate, phenyl salicylate, salacetamide, salicylamide O-acetic acid, salsalate and sulfasalazine. Structurally related salicylic acid derivatives having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more propionic acid derivatives, prodrug esters or pharmaceutically acceptable salts thereof. The propionic acid derivatives, prodrug esters and pharmaceutically acceptable salts thereof comprise: alminoprofen, benoxaprofen, bucloxic acid, carprofen, dexindoprofen, fenoprofen, flynoxaprofen, fluprofen, flurbiprofen, furcloprofen, ibuprofen, ibuprofen aluminum, ibuproxam, indoprofen, isoprofen, ketoprofen, loxoprofen, miroprofen, naproxen, oxaprozin, piketoprofen, pimeprofen, pirprofen, pranoprofen, protizinic acid, pyridoxiprofen, suprofen, tiaprofenic acid and tioxaprofen. Structurally related propionic acid derivatives having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more acetic acid derivatives, prodrug esters or pharmaceutically acceptable salts thereof. The acetic acid derivatives, prodrug esters and pharmaceutically acceptable salts thereof comprise: acemetacin, alclofenac, amfenac, bufexamac, cinmetacin, clopirac, delmetacin, diclofenac sodium, etodolac, felbinac, fenclofenac, fenclorac, fenclozic acid, fentiazac, furofenac, glucametacin, ibufenac, indomethacin, isofezolac, isoxepac, lonazolac, metiazinic acid, oxametacin, oxpinac, pimetacin, proglumetacin, sulindac, talmetacin, tiaramide, tiopinac, tolmetin, zidometacin and zomepirac. Structurally related acetic acid derivatives having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more fenamic acid derivatives, prodrug esters or pharmaceutically acceptable salts thereof. The fenamic acid derivatives, prodrug esters and pharmaceutically acceptable salts thereof comprise: enfenamic acid, etofenamate, flufenamic acid, isonixin, meclofenamic acid, meclofenamate sodium, medofenamic acid, mefanamic acid, niflumic acid, talniflumate, terofenamate, tolfenamic acid and ufenamate. Structurally related fenamic acid derivatives having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more carboxylic acid derivatives, prodrug esters or pharmaceutically acceptable salts thereof. The carboxylic acid derivatives, prodrug esters and pharmaceutically acceptable salts thereof which can be used comprise: clidanac, diflynisal, flufenisal, inoridine, ketorolac and tinoridine. Structurally related carboxylic acid derivatives having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more butyric acid derivatives, prodrug esters or pharmaceutically acceptable salts thereof. The butyric acid derivatives, prodrug esters and pharmaceutically acceptable salts thereof comprise: bumadizon, butibufen, fenbufen and xenbucin. Structurally related butyric acid derivatives having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more oxicams, prodrug esters or pharmaceutically acceptable salts thereof. The oxicams, prodrug esters and pharmaceutically acceptable salts thereof comprise: droxicam, enolicam, isoxicam, piroxicam, sudoxicam, tenoxicam and 4-hydroxyl-1,2-benzothiazine 1,1-dioxide 4-(N-phenyl)-carboxamide. Structurally related oxicams having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra

product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more pyrazoles, prodrug esters or pharmaceutically acceptable salts thereof. The pyrazoles, prodrug esters and pharmaceutically acceptable salts thereof which may be used comprise: difenamizole and epirizole. Structurally related pyrazoles having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more pyrazolones, prodrug esters or pharmaceutically acceptable salts thereof. The pyrazolones, prodrug esters and pharmaceutically acceptable salts thereof which may be used comprise: apazone, azapropazone, benzpiperylon, feprazone, mofebutazone, morazone, oxyphenbutazone, phenylbutazone, pipebuzone, propylphenazone, ramifenazone, suxibuzone and thiazolinobutazone. Structurally related pyrazalones having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more of the following NSAIDS: ε-acetamidocaproic acid, S-adenosylmethionine, 3-amino-4-hydroxybutyric acid, amixetrine, anitrazafen, antrafenine, bendazac, bendazac lysinate, benzydamine, beprozin, broperamole, bucolome, bufezolac, ciproquazone, cloximate, dazidamine, deboxamet, detomidine, difenpiramide, difenpyramide, difisalamine, ditazol, emorfazone, fanetizole mesylate, fenflumizole, floctafenine, flumizole, flunixin, fluproquazone, fopirtoline, fosfosal, guaimesal, guaiazolene, isonixirn, lefetamine HCl, leflunomide, lofemizole, lotifazole, lysin clonixinate, meseclazone, nabumetone, nictindole, nimesulide, orgotein, orpanoxin, oxaceprolm, oxapadol, paranyline, perisoxal, perisoxal citrate, pifoxime, piproxen, pirazolac, pirfenidone, proquazone, proxazole, thielavin B, tiflamizole, timegadine, tolectin, tolpadol, tryptamid and those designated by company code number such as 480156S, AA861, AD1590, AFP802, AFP860, AI77B, AP504, AU8001, BPPC, BW540C, CHINOIN 127, CN100, EB382, EL508, F1044, FK-506, GV3658, ITF182, KCNTEI6090, KME4, LA2851, MR714, MR897, MY309, ON03144, PR823, PV102, PV108, R830, RS2131, SCR152, SH440, SIR133, SPAS510, SQ27239, ST281, SY6001, TA60, TAI-901 (4-benzoyl-1-indancarboxylic acid), TVX2706, U60257, UR2301 and WY41770. Structurally related NSAIDs having similar analgesic and anti-inflammatory properties to the above NSAIDs are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more corticosteroids, prodrug esters or pharmaceutically acceptable salts thereof for the treatment of IL-1-mediated diseases, as defined above, including acute and chronic inflammation such as rheumatic diseases (e.g., lyme disease, juvenile (rheumatoid) arthritis, osteoarthritis, psoriatic arthritis, rheumatoid arthritis and staphylococcal-induced ("septic") arthritis); graft versus host disease and multiple sclerosis. Corticosteroids, prodrug esters and pharmaceutically acceptable salts thereof include hydrocortisone and compounds which are derived from hydrocortisone, such as 21-acetoxypregnenolone, alclomerasone, algestone, amcinonide, beclomethasone, betamethasone, betamethasone valerate, budesonide, chloroprednisone, clobetasol, clobetasol propionate, clobetasone, clobetasone butyrate, clocortolone, cloprednol, corticosterone, cortisone, cortivazol, deflazacon, desonide, desoximerasone, dexamethasone, diflorasone, diflucortolone, difluprednate, enoxolone, fluazacort, flucloronide, flumethasone, flumethasone pivalate, flunisolide, flucinolone acetonide, fluocinonide, fluorocinolone acetonide, fluocortin butyl, fluocortolone, fluorocortolone hexanoate, diflucortolone valerate, fluorometholone, fluperolone acetate, fluprednidene acetate, fluprednisolone, flurandenolide, formocortal, halcinonide, halometasone, halopredone acetate, hydrocortamate, hydrocortisone, hydrocortisone acetate, hydrocortisone butyrate, hydrocortisone phosphate, hydrocortisone 21-sodium succinate, hydrocortisone tebutate, mazipredone, medrysone, meprednisone, methylprednicolone, mometasone furoate, paramethasone, prednicarbate, prednisolone, prednisolone 21-diedryaminoacetate, prednisolone sodium phosphate, prednisolone sodium succinate, prednisolone sodium 21-m-sulfobenzoate, prednisolone sodium 21-stearoglycolate, prednisolone tebutate, prednisolone 21-trimethylacetate, prednisone, prednival, prednylidene, prednylidene 21-diethylaminoacetate, tixocortol, triamcinolone, triamcinolone acetonide, triamcinolone benetonide and triamcinolone hexacetonide. Structurally related corticosteroids having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more slow-acting antirheumatic drugs (SAARDs) or disease modifying antirheumatic drugs (DMARDS), prodrug esters or pharmaceutically acceptable salts thereof for the treatment of IL-1-mediated diseases, as defined above, including acute and chronic inflammation such as rheumatic diseases (e.g., lyme disease, juvenile (rheumatoid) arthritis, osteoarthritis, psoriatic arthritis, rheumatoid arthritis and staphylococcal-induced ("septic") arthritis); graft versus host disease and multiple sclerosis. SAARDs or DMARDS, prodrug esters and pharmaceutically acceptable salts thereof comprise: allocupreide sodium, auranofin, aurothioglucose, aurothioglycanide, azathioprine, brequinar sodium, bucillamine, calcium 3-aurothio-2-propanol-1-sulfonate, chlorambucil,

chloroquine, clobuzarit, cuproxoline, cyclophosphamide, cyclosporin, dapsone, 15-deoxyspergualin, diacerein, glucosamine, gold salts (e.g., cycloquine gold salt, gold sodium thiomalate, gold sodium thiosulfate), hydroxychloroquine, hydroxyurea, kebuzone, levamisole, lobenzarit, melittin, 6-mercaptopurine, methotrexate, mizoribine, mycophenolate mofetil, myoral, nitrogen mustard, D-penicillamine, pyridinol imidazoles such as SKNF86002 and SB203580, rapamycin, thiols, thymopoietin and vincristine. Structurally related SAARDs or DMARDs having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more COX2 inhibitors, their prodrug esters or pharmaceutically acceptable salts thereof for the treatment of IL-1-mediated diseases, as defined above, including acute and chronic inflammation. Examples of COX2 inhibitors, prodrug esters or pharmaceutically acceptable salts thereof include, for example, celecoxib. Structurally related COX2 inhibitors having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more antimicrobials, prodrug esters or pharmaceutically acceptable salts thereof for the treatment of IL-1-mediated diseases, as defined above, including acute and chronic inflammation. Antimicrobials, prodrug esters and pharmaceutically acceptable salts thereof include, for example, ampicillin, amoxycillin, aureomicin, bacitracin, ceftazidime, ceftriaxone, cefotaxime, cephachlor, cephalexin, cephradine, ciprofloxacin, clavulanic acid, cloxacillin, dicloxacillan, erythromycin, flucloxacillan, gentamicin, gramicidin, methicillan, neomycin, oxacillan, penicillin and vancomycin. Structurally related antimicrobials having similar analgesic and anti-inflammatory properties are also intended to be encompassed by this group.

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more TNF inhibitors for the treatment of IL-1-mediated diseases, as defined above, including acute and chronic inflammation such as rheumatic diseases (e.g., lyme disease, juvenile (rheumatoid) arthritis, osteoarthritis, psoriatic arthritis, rheumatoid arthritis and staphylococcal-induced ("septic") arthritis); brain injury as a result of trauma, epilepsy, hemorrhage or stroke; and graft versus disease. Such TNF inhibitors include compounds and proteins which block in vivo synthesis or extracellular release of TNF. In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with any of one or more of the following TNF inhibitors: TNF binding proteins (soluble TNF receptor Type I and soluble TNF receptor Type II ("sTNFRs")), anti-TNF antibodies, granulocyte colony stimulating factor; thalidomide; BN 50730; tenidap; E 5531; tiapafant PCA 4248; nimesulide; panavir; rolipram; RP 73401; peptide T; MDL 201,449A; (1R,3S)-Cis-1-[9-(2,6-diaminopurinyl)]-3-hydroxy-4-cyclopentene hydrochloride; (1R,3R)-trans-1-[9-(2,6-diamino)purine]-3-acetoxycyclopentane; (1R,3R)-trans-1-[9-adenyl)-3-azidocyclopentane hydrochloride and (1R,3R)-trans-1-[6-hydroxy-purin-9-yl)-3-azidocyclopentane.

TNF binding proteins are disclosed in the art (EP 308 378, EP 422 339, GB 2 218 101, EP 393 438, WO 90/13575, EP 398 327, EP 412 486, WO 91/03553, EP 418 014, JP 127,800/1991, EP 433 900, U.S. Pat. No. No. 5,136,021, GB 2 246 569, EP 464 533, WO 92/01002, WO 92/13095, WO 92/16221, EP 512 528, EP 526 905, WO 93/07863, EP 568 928, WO 93/21946, WO 93/19777, EP 417 563, PCT International Application No. US97/12244, filed on Jul. 9, 1997 by Fisher, Edwards and Kieft, entitled on the PCT Application transmittal letter as "TRUNCATED SOLUBLE TUMOR NECROSIS FACTOR TYPE-I AND TYPE-II RECEPTORS" the disclosures of which are hereby incorporated by reference).

For example, EP 393 438 and EP 422 339 teach the amino acid and nucleic acid sequences of a "30 kDa TNF inhibitor" (also known as the p55 receptor) and a "40 kDa inhibitor" (also known as the p75 receptor) as well as modified forms thereof (e.g., fragments, functional derivatives and variants). EP 393 438 and EP 422 339 also disclose methods for isolating the genes responsible for coding the inhibitors, cloning the gene in suitable vectors and cell types and expressing the gene to produce the inhibitors. Additionally, polyvalent forms (i.e., molecules comprising more than one active moiety) of the above-described TNF inhibitors have also been disclosed. In one embodiment, the polyvalent form may be constructed, for example, by chemically coupling at least one TNF inhibitor and another moiety with any clinically acceptable linker, for example polyethylene glycol (WO 92/16221 and WO 95/34326), by a peptide linker (Neve et al. (1996), *Cytokine,* 8(5):365–370) by chemically coupling to biotin and then binding to avidin (WO 91/03553) and, finally, by constructing chimeric antibody molecules (U.S. Pat. No. 5,116,964, WO 89/09622, WO 91/16437 and EP 315062).

Anti-TNF antibodies include MAK 195F Fab antibody (Holler et al.(1993), 1st International Symposium on Cytokines in Bone Marrow Transplantation, 147); CDP 571 anti-TNF monoclonal antibody (Rankin et al. (1995), *British Journal of Rheumatology,* 34:334–342); BAY X 1351 murine anti-tumor necrosis factor monoclonal antibody (Kieft et al. (1995), 7th European Congress of Clinical Microbiology and Infectious Diseases, 9); CenTNF cA2 anti-TNF monoclonal antibody (Elliott et al. (1994), *Lancet,* 344:1125–1127 and Elliott et al. (1994), *Lancet,* 344:1105–1110).

In a specific embodiment, the present invention is directed to the use of an IL-1 inhibitor (e.g., preferably IL-1ra

product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra) in combination (pretreatment, post-treatment or concurrent treatment) with the soluble recombinant human Fas antigen or recombinant versions thereof for the treatment of IL-1-mediated diseases, as defined above, including acute and chronic inflammation such as rheumatic diseases (e.g., lyme disease, juvenile (rheumatoid) arthritis, osteoarthritis, psoriatic arthritis, rheumatoid arthritis and staphylococcal-induced ("septic") arthritis); and graft versus host disease. Soluble recombinant human Fas antigen, and variants thereof such as a fas fusion protein, methods for isolating the genes responsible for coding the soluble recombinant human Fas antigen, methods for cloning the gene in suitable vectors and cell types, and methods for expressing the gene to produce the inhibitors are known (WO 96/20206 and Mountz et al., *J. Immunology,* 155:4829–4837, the disclosures of which are hereby incorporated by reference).

The above is by way of example and does not preclude other treatments to be used concurrently with these anti-arthritic compounds that are known by those skilled in the art or that could be arrived at by those skilled in the art using the guidelines set forth in this specification.

It is especially advantageous to formulate compositions of the additional anti-inflammatory compounds in dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form" as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated, each unit containing a predetermined quantity of additional anti-inflammatory compounds calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coating, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like which are compatible with the active ingredient and with the mode of administration and other ingredients of the formulation and not deleterious to the recipient. The use of such media and agents is well known in the art (*Remington's Pharmaceutical Sciences,* 18th Ed. (1990), Mack Publishing Co., Easton, Pa. 18042, pages 1435–1712). An exemplary pharmaceutically acceptable carrier is phosphate buffered saline. Supplementary active ingredients can also be incorporated into the compositions.

For oral therapeutic administration, the additional anti-inflammatory compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixers, suspensions, syrups, wafers and the like, or it may be incorporated directly with the food in the diet. The tablets, troches, pills, capsules and the like may also contain the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, alginic acid and the like; a lubricant such as magnesium stearate; a sweetening agent such as sucrose, lactose or saccharin; or a flavoring agent such as peppermint, oil of wintergreen or cherry or orange flavoring. When the dosage unit form is a capsule, it may contain, in addition to material of the above type, a liquid carrier. Various other materials may be present as a coating or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills or capsules may be coated with shellac, sugar or both. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the additional anti-inflammatory compound may be incorporated into a controlled-release preparation and formulation. The amount of the additional anti-inflammatory compound in such a therapeutically useful composition is such that a suitable dosage will be obtained.

For parenteral therapeutic administration, each anti-inflammatory compound may be incorporated with a sterile injectable solution. The sterile injectable solution may be prepared by incorporating the additional anti-inflammatory compound in the required amount in an appropriate pharmaceutically acceptable carrier, with various other ingredients enumerated below (required), followed by filtered sterilization. In the case of dispersions, each may be prepared by incorporating the additional anti- inflammatory compound into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile injectable solutions, each may be prepared by incorporating a powder of at least one additional anti-inflammatory compound and, optionally, any additional desired ingredient from a previously sterile-filtered solution thereof, wherein the powder is prepared by any suitable technique (e.g., vacuum drying and freeze drying).

The specific dose of the additional anti-inflammatory compound is calculated according to the approximate body weight or surface area of the patient. Other factors in determining the appropriate dosage can include the acute or chronic inflammatory disease or condition to be treated or prevented, the severity of the disease, the route of administration, and the age, sex and medical condition of the patient. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above-mentioned formulations is routinely made by those skilled in the art. Dosages can also be determined through the use of known assays for determining dosages used in conjunction with appropriate dose-response data.

Thus, for example, it is within the scope of the invention that doses of the additional anti-inflammatory compounds selected for treating a particular acute or chronic inflammatory disease can be varied to achieve a desired therapeutic effect. Where one of the additional anti-inflammatory compounds has side effects, it can be given to patients during alternate treatment periods of combination therapy. For example, chronic methotrexate treatment is associated with gastrointestinal, hepatic, bone marrow and pulmonary toxicity (Sandoval et al. (1995), *British Journal of Rheumatology,* 34:49–56).

Tests for monitoring the improvement of a disease can include specific tests directed, for example, to the determination of systemic response to inflammation, which include the erythrocyte sedimentation rate (ESR) and acute phase reactants (APR). Observations are made of the swelling, etc. of the afflicted body parts. Improvement in stiffness, and grip (where applicable), and reduction in pain of the patient is also observed. If the patient's condition is stable, he is re-treated at the same dosage weekly and is evaluated weekly. Provided the patient's condition is stable, the treat-

ment may be continued. After six months of treatment, anatomical changes of the skeleton are determined by radiologic imaging, for example by X-radiography.

At the end of each period, the patient is again evaluated. Comparison of the pre-treatment and post-treatment radiological assessment, ESR and APR indicates the efficacy of the treatments. According to the efficacy of the treatments and the patient's condition, the dosage may be increased or maintained constant for the duration of treatment.

Preferably, the present invention is directed to a method comprising the use of one of the following combinations to treat or prevent an acute or chronic inflammatory disease and condition, as defined above, such as rheumatic diseases (e.g., lyme disease, juvenile (rheumatoid) arthritis, osteoarthritis, psoriatic arthritis, rheumatoid arthritis and staphylococcal-induced ("septic") arthritis) and the symptoms associated therewith: I IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan), and methotrexate; IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan), and any one or more of methotrexate, sulphasalazine and hydroxychloroquine; IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan), methotrexate and hydroxychloroquine; IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan), methotrexate and sulphasalazine; and IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan), methotrexate and a TNF inhibitor, preferably sTNFRs.

In a specific preferred embodiment, the method comprises the administration (e.g., intra-articular, subcutaneous or intramuscular) of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan), the citrate buffer formulation or the phosphate buffer formulation) in combination (pretreatment, post-treatment or concurrent treatment) with methotrexate and/or sTNFRs to treat arthritis (e.g., osteoarthritis, psoriatic arthritis and/or rheumatoid arthritis) and the symptoms associated therewith.

In a specific preferred embodiment, the method comprises the administration (e.g., intravenous or intraventricular) of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan), the citrate buffer formulation or the phosphate buffer formulation) in combination (pretreatment, post-treatment or concurrent treatment) with tissue plasminogen activator and/or sTNFRs to treat brain injury as a result of trauma, epilepsy, hemorrhage or stroke, each of which may lead to neurodegeneration.

In a specific preferred embodiment, the method comprises the administration (e.g., subcutaneous or intramuscular) of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan), the citrate buffer formulation or the phosphate buffer formulation) in combination (pretreatment, post-treatment or concurrent treatment) with one or more of a corticosteroid, cyclosporin or an interferon (e.g., alpha interferon, beta interferon, gamma interferon and consensus interferon) and/or sTNFRs to treat multiple sclerosis.

In a specific preferred embodiment, the method comprises the administration (e.g., intravenous) of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan), the citrate buffer formulation or the phosphate buffer formulation) in combination (pretreatment, post-treatment or concurrent treatment) with one or more of methotrexate, a corticosteroid, FK506, cyclosporin, a soluble fas protein and/or sTNFRs to treat graft versus host rejection.

In a specific preferred embodiment, the method comprises the administration (e.g., subcutaneous or intramuscular) of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan), the citrate buffer formulation or the phosphate buffer formulation) in combination (pretreatment, post-treatment or concurrent treatment) with G-CSF and/or sTNFRs to treat inflammatory bowel disease.

In a specific preferred embodiment, the method comprises the administration (e.g., subcutaneous or intramuscular) of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan), the citrate buffer formulation or the phosphate buffer formulation) in combination (pretreatment, post-treatment or concurrent treatment) with interferon (e.g., alpha interferon, beta interferon, gamma interferon and consensus interferon) to treat multiple myeloma or myelogenous (e.g., AML and CML) and other leukemias.

In a specific preferred embodiment, the method comprises the administration (e.g., subcutaneous, intraventricular or intrathecal) of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan)) in combination (pretreatment, post-treatment or concurrent treatment) with an NSAID (e.g., indomethacin) and/or sTNFRs to treat Alzheimer's disease.

In a specific preferred embodiment, the method comprises the administration (e.g., local injection, subcutaneous or intramuscular) of an IL-1 inhibitor (e.g., preferably IL-1ra product (including, but not limited to, rhuIL-1ra Fc fusion proteins) and more preferably IL-1ra, optionally formulated with a controlled release polymer (e.g., hyaluronan)) to treat temporal mandibular joint disease.

The following examples are included to more fully illustrate the present invention. It is understood that modifica-

tions can be made in the procedures set forth without departing from the spirit of the invention.

EXAMPLES

Standard methods for many of the procedures described in the following examples, or suitable alternative procedures, are provided in widely recognized manuals of molecular biology such as, for example, Sambrook et al., *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory Press (1987) and Ausabel et al., *Current Protocols in Molecular Biology,* Greene Publishing Associates/Wiley Interscience, New York (1990). All chemicals were either analytical grade or USP grade.

Example 1

Sample Preparation: An *E. coli*-derived human recombinant IL-1 receptor antagonist (rhuIL-1ra), prepared generally in accordance with the teachings of U.S. Pat. No. 5,075,222, was formulated in 10 millimolar sodium citrate, 140 millimolar sodium chloride, 0.5 millimolar EDTA, 0.1% polysorbate (w/w) in water, pH 6.5 (CSEP). Syringes containing the formulated IL-1ra were then each attached by means of a stopcock to a syringe containing one of the following controlled release materials: H-10™ hylan fluid (Biomatrix, Inc., Ridgefield, Inc.), a cross-linked hyaluronic acid (Mr^3 4×10^6) as either a dry powder or dry powder reconstituted in PBS; hyaluronic acid (Mr^3 570,000) in PBS derived from cultures of *Streptococcus zooepidemicus* (catalog #H9390, Sigma, Inc., St.Louis, Mo.) as a dry powder; polyvinyl pyrrolidone (Mr 1.3×10^6) (catalog #43, 719-0, Aldridge Chemical Co., Inc., Milwaukee, Wis.) as a dry powder; and carboxymethyl cellulose (carboxymethyl cellulose (catalog #06139, Polysciences, Inc., Warrington, Pa.) as a dry powder. The IL-1ra was then admixed with the control release material by injecting the rhuIL-1ra solution into the syringe containing the hyaluronic acid and injecting the contents back and forth several times to ensure mixing.

Accordingly, the following formulations were prepared: (1) IL-1ra (100 mg/ml)/2% H-10™ hylan; (2) IL-1ra (100 mg/ml)/1% hyaluronic acid; (3) IL-1ra (100 mg/ml)/0.5% H-10™ hylan; (4) IL-1ra (100 mg/ml)/2% hyaluronic acid, (5) IL-1ra (100 mg/ml)/4% polyvinyl pyrrolidone and (6) IL-1ra (100 mg/ml)/3% carboxymethyl cellulose.

The various formulations were injected subcutaneously into female Lewis rats (200–250 g, Charles River, Portage, Mich.). At various times after injection, blood was drawn via catheters inserted into the jugular veins of the animals. The blood was centrifuged to remove blood cells and the remaining plasma was assayed for IL-1ra using an ELISA kit (Quantikine™ human IL-1ra immunoassay, R&D Systems, Minneapolis, Minn.) according to the manufacturer's guidelines. The data are expressed as plasma IL-1ra (μg/ml IL-1ra in plasma) vs. time after injection, as shown in Table 2 and FIG. **1**.

TABLE 2

	Plasma IL-1ra (μg/ml) after Subcutaneous Injection						
Time after Injection (Hours)	IL-1ra alone	IL-1ra (100 mg/ml)/ H-10 ™ hylan (2% w/v)	IL-1ra (100 mg/ml)/ H-10 ™ hylan (1% w/v)	IL-1ra (100 mg/ml)/ H-10 ™ hylan (0.5% w/v)	IL-1ra (100 mg/ml)/ hyaluronic acid (Mr^3 5.7×10^5) (2% w/v)	IL-1ra (100 mg/ml)/ PVP (4% w/v)	IL-1ra (100 mg/ml)/ CMC (3% w/v)
0	0.0682 ± 0.023	0.027 ± 0.012	0.007 ± 0.002	0.02 ± 0.008	0.007 ± 0.004	0.502 ± 0.311	ND*
0.167	4.186 ± 0.082	2.515 ± 0.429	1.65 ± 0.147	3.085 ± 0.492	2.341 ± 0.278	1.623 ± 0.247	ND*
0.25	ND*	ND*	ND*	ND*	ND*	ND*	1.93 ± 0.58
0.5	7.658 ± 0.267	4.429 ± 0.567	4.096 ± 0.395	7.167 ± 0.656	4.489 ± 0.309	2.642 ± 0.712	2.46 ± 0.57
1	13.659 ± 2.21	5.98 ± 0.825	5.881 ± 0.865	9.23 ± 0.417	6.217 ± 0.551	4.809 ± 1.936	3.54 ± 0.56
2	9.813 ± 1.135	6.201 ± 0.697	6.708 ± 0.534	11.225 ± 0.759	5.704 ± 0.714	5.461 ± 0.899	5.05 ± 0.94
4	5.252 ± 0.055	6.12 ± 0.834	5.532 ± 0.852	9.225 ± 0.948	6.495 ± 0.945	7.248 ± 1.186	4.49 ± 0.62
8	1.082 ± 0.142	3.354 ± 0.279	4.744 ± 0.716	5.146 ± 0.449	3.438 ± 0.546	8.447 ± 2.406	4.27 ± 0.53
12	0.043 ± 0.01	2.024 ± 0.231	1.896 ± 0.1	ND*	4.559 ± 0.322	2.231 ± 0.825	2.37 ± 0.22
24	0.01 ± 0.003	0.345 ± 0.073	0.252 ± 0.059	0.07 ± 0.016	0.112 ± 0.016	0.04 ± 0.005	0.46 ± 0.08
48	ND*	0.061 ± 0.032	0.012 ± 0.005	0.01 ± 0.004	0.013 ± 0.006	ND*	0.19 ± 0.12
72	ND*	ND*	0.008 ± 0.003	0.008 ± 0.003	0.004 ± 0.003	ND*	0.85 ± 0.42

*No data presented.

As shown in Table 2 and FIG. **1**, incorporation of IL-1ra into hyaluronan, polyvinyl pyrrolidone and carboxymethyl cellulose leads to prolonged elevation of IL-1ra plasma levels as compared to IL-1ra administered alone.

Example 2

IL-1ra in CSEP was radiolabeled with Na[^{125}I], and then incorporated into H-10™ hylan fluid (2% final), as described

above. Radioactive IL-1ra or IL-1ra/H-10™ hylan mixtures were injected intraarticularly into the hind knees of guinea pigs (Charles River, Portage, Mich.). At various times after injection, the animals were sacrificed and the knee joints removed and counted in a gamma counter, as described in van Lent et al, (1989), *J. Rheumatol.,* 16:1295–1303. The amount of IL-1ra remaining in the joints at each time point is shown in FIG. **2**. The intraarticular half lives of IL-1ra in three different hyaluronan formulations were calculated from graphs such as FIG. **2**, and are shown in Table 3.

TABLE 3

Joint Half-life of IL-1ra Formulations in Guinea Pigs after Intraarticular Injection

Formulation[a]	Ratio[b]	Half-life (hours
IL-1ra alone	NA	1.36
IL-1ra/hyaluronan	90/10	3.54
IL-1ra/hyaluronan	80/20	2.45
IL-1ra/hyaluronan	50/50	1.45

[a]IL-1ra concentration 100 mg/ml. When applicable, hyaluronan concentration 2% (w/v).
[b]Ratio of fluid (non-crosslinked) to gel (cross-linked) hyaluronan in formulation.

As shown in Table 3 and FIG. **2**, incorporation of IL-1ra into hyaluronan leads to prolonged retention of IL-1ra in knee joints after intraarticular administration. The degree of retention can be controlled by the ratio of crosslinked (gel) to non-crosslinked (fluid) hyaluronan in the formulation.

Example 3

IL-1ra in CSEP or a formulation of IL-1ra (100 mg/ml)/2% H-10™ hylan, as described above, was injected intraarticularly into the hind knees of rabbits (Charles River, Portage, Mich.). At various times after injection, the animals were sacrificed and the knees lavaged with PBS to recover the synovial fluid. The concentration of IL-1ra (μg/ml) in the recovered synovial fluid was determined by ELISA (Quantikine™, human IL-1ra immunoassay, R&D Systems) according to the manufacturer's specifications. The data are shown in Table 4 and FIG. **3**.

TABLE 4

Joint Half-life of IL-1ra Formulations in Hind Knees Rabbits after Intraarticular Injection

Time after Injection (Hours)	IL-1ra alone	IL-1ra (100 mg/ml)/hyaluronan (2% w/v)
0.5	2280	2440
0.5	11000	6200
0.5	5000	2410
0.5	8090	ND*
1	1400	5150
1	3450	6830
1	3090	7180
1	1840	2620
4	56.98	224
4	31.24	1600
4	62.43	3250
4	ND*	237
8	0.0641	575
8	0.0312	55.98
8	ND*	125
8	ND*	ND*
24	ND*	0.5644
24	ND*	0.1539
24	ND*	0.8852

*No data presented.

This data shows that the hyaluronan formulation of IL-1ra of prolonged release of intact IL-1ra into the synovial fluid after intraarticular injection.

Example 4

Female Lewis rats (200–250 g, Charles River, Portage, Mich.) were immunized on day 0 and day 7 with bovine type II collagen (Elastin Products, Owensville, Mo.). Arthritis developed starting on days 12–13. The rats (8 animals/group) were injected intraarticularly with either H-10™ hylan fluid in CSEP (50 μl/knee; 1 mg hyaluronan total) or IL-1ra (100 mg/ml)/2% H-10™ hylan (50 μl/knee; 5 mg IL-1ra/1 mg hyaluronan) on days 15 and 18 after initial immunization. An arthritis control group received no injection. On day 20, after initial immunization, the rats were sacrificed and the knee joints collected for histologic evaluation of disease severity. As shown in Table 5 and FIG. **4**: (a) treatment with IL-1ra significantly suppressed cartilage and bone damage and had a modest effect on synovitis; (b) total joint damage was reduced by 70% compared to controls and (c) treatment with hyaluronan alone had no beneficial effects in comparison to disease controls.

TABLE 5

FORMULATIONS	IL-1ra Concentration in Synovial Fluid after Intraarticular Injection: SYNOVITIS	PANNUS	TOTAL CARTILAGE	BONE DAMAGE	JOINT TOTAL
Untreated	3.25 ± 0.14	1.5 ± 0.16	17.69 ± 2.27	1.44 ± 0.16	23.88 ± 2.57
hyaluronan (2% w/v)	2.88 ± 0.39	1.44 ± 0.22	16.88 ± 3.14	1.31 ± 0.25	22.5 ± 3.87
IL-1ra (100 mg/ml)/ hyaluronan (2% w/v)	2.06 ± 0.28	0.5 ± 0.18	4.69 ± 1.77	0.19 ± 0.14	7.53 ± 2.33

Example 5

Female Lewis rats (200–250 g, Charles River, Portage, Mich.) were given intradermal injections of 2 mg/ml of bovine type II collagen (Elastin Products, Owensville, Mo.) in incomplete Freund's Adjuvant (Difco Laboratories, Inc., Ann Arbor, Mich.) at the base of the tail and over the back in 3 sites (250 μl divided) on day 0 and day 7. On day 12 they were given an intraperitoneal injection of 3 mg/kg of endotoxin (LPS type L-3129, Sigma). Onset of arthritis occurred over the next 5 days and as rats developed disease they were randomized to study groups (6–8/group) and treatment was initiated. The rats were treated for 6 days (subcutaneous injections of IL-1ra (100 mg/ml)/2% H-10™ hylan fluid, as defined above, in dorsum of the back) and then sacrificed on day 7 of arthritis for assessment of paw weights and tissue collection.

Caliper measurements of ankle joint width were done prior to onset of arthritis, on the day of randomization and on each subsequent study day until termination of the study on arthritis day 7. The data were then expressed as area under the curve for purposes of determining the percent inhibition from controls over the duration of the arthritis. At termination, the tibiotarsal joint was transected at the level of the medial and lateral malleolus for determination of final paw weights as another measure of inflammation. Ankle joints were then collected into formalin for histopathologic evaluation.

Histopathology: Ankle joints were collected into 10% neutral buffered formalin for at least 24 hours prior to placement in a Surgipath decalcifier I (Surgipath, Grayslake, Ill.) for approximately 1 week. When decalcification was complete, the digits were trimmed and the ankle joint was transected in the longitudinal plane to give approximately equal halves. These were processed for paraffin embedding, sectioned and stained with hematoxylin and eosin for general evaluation of inflammation and bone damage and stained with toluidine blue for specific evaluation of cartilage changes according to the following criteria:

Inflammation

0=Normal

1=Minimal infiltration of inflammatory cells in periarticular tissue

2=Mild infiltration

3=Moderate infiltration with moderate edema

4=Marked infiltration with marked edema

5=Severe infiltration with severe edema

Cartilage Damage

0=Normal

1=Minimal to mild loss of toluidine blue staining with no obvious chondrocyte loss or collagen disruption

2=Mild loss of toluidine blue staining with focal mild (superficial) chondrocyte loss and/or collagen disruption

3=Moderate loss of toluidine blue staining with multifocal moderate (depth to middle zone) chondrocyte loss and/or collagen disruption

4=Marked loss of toluidine blue staining with multifocal marked (depth to deep zone) chondrocyte loss and/or collagen disruption

5=Severe diffuse loss of toluidine blue staining with multifocal severe (depth to tide mark) chondrocyte loss and/or collagen disruption

Bone Resorption

0=Normal

1=Minimal small areas of resorption, not readily apparent on low magnification, rare osteoclasts

2=Mild has more numerous areas of resorption, not readily apparent on low magnification, osteoclasts more numerous

3=Moderate has obvious resorption of medullary trabecular and cortical bone without full thickness defects in cortex, loss of some medullary trabeculae, lesion apparent on low magnification, osteoclasts more numerous

4=Marked has full thickness defects in cortical bone, often with distortion of profile of remaining cortical surface, marked loss of medullary, numerous osteoclasts,

5=Severe has full thickness defects in cortical bone, often with distortion of profile of remaining cortical surface, marked loss of medullary bone of distal tibia, numerous osteoclasts, resorption also present in smaller tarsal bones

Statistical Analysis: Clinical data for ankle width was analyzed by determining the area under the dosing curve with subsequent analysis of variance. Paw weights (mean±SE) for each group were analyzed for differences using the Student's T Test.

In contrast to the lack of efficacy when single daily 100 mg/kg doses of IL-1ra in CSEP were given, administration of single daily (QD) subcutaneous (SQ) doses of IL-1ra (100 mg/ml)/2% H-10™ hylan fluid resulted in 62% inhibition of paw swelling over time and 74% inhibition of final paw weights (FIGS. 6 and 7). These results clearly demonstrate the superior clinical effects of daily dosing of IL-1ra (100

mg/ml)/2% H-110™ hylan fluid vs. IL-1ra in CSEP. In addition, histologic analysis of ankle joint sections revealed marked decreases in inflammation, pannus formation, and cartilage and bone damage in rats treated with IL-1ra (100 mg/ml)/2% H-10™ hylan fluid but not IL-1ra in CSEP (FIG. **8**).

After confirming that single daily subcutaneous doses of IL-1ra (100 mg/ml)/2% H-10™ hylan fluid were able to modulate disease progression, studies were done to determine the duration of effect. Rats treated with IL-1ra (100 mg/ml)/2% H-10™ hylan fluid every day had 53% inhibition of paw swelling over time and 78% inhibition of final paw weights (FIGS. **9** and **10**). Arthritic rats treated every other day with IL-1ra (100 mg/ml)/2% H-10™ hylan fluid had 35% inhibition of paw swelling over time and 62% inhibition of final paw weights. Arthritic rats treated with IL-1ra (100 mg/ml)/2% H-10™ hylan fluid every third day had 27% inhibition (nonsignificant) of swelling over time and 19% inhibition of paw weights. These results again demonstrate the importance of maintaining minimal blood levels of at least 200 ng/ml during the period of time in which IL-1 is operative in the pathogenesis in the model. Blocking the IL-1 receptor intermittently results in less efficacy. Rats treated every third day, were dosed on day 1 and day 4 of arthritis. Interestingly, caliper measurements done 24 hrs. post dosing (day 2 and day 5) indicate suppression of arthritis progression (FIG. **9**). However, measurements taken 2 or 3 days post dosing prior to rats being given their next dose, reflect disease progression, presumably as a result of the less than optimal blood levels during that period of time.

Example 6

Expression of rhuIL-1ra Fc fusion proteins in *E. coli.*

A. Recombinant Human IL-1ra

The synthetic NdeI-HindIII IL-1ra gene fragment (shown below) was enzymatically cleaved from another expression vector and ligated to the same sites of expression vector pAMG21 (European Patent Application No. 96309363.8).

Map of Synthetic IL-1ra NdeI to HindIII fragment:

```
NdeI                        SacI
 |                           |
   catatgcgaccgtccggccgtaagagctccaaaatgcaggctttccgtatctgggacgtt                (SEQ ID NO 3
 1 ---------+---------+---------+---------+---------+---------+     60
    M  R  P  S  G  R  K  S  S  K  M  Q  A  F  R  I  W  D  V                  (SEQ ID NO 4)

   aaccagaaaaccttctacctgcgcaacaaccagctggttgctggctacctgcagggtccg
61 ---------+---------+---------+---------+---------+---------+    120
   N  Q  K  T  F  Y  L  R  N  N  Q  L  V  A  G  Y  L  Q  G  P

   aacgttaacctggaagaaaaaatcgacgttgtaccgatcgaaccgcacgctctgttcctg
121 ---------+---------+---------+---------+---------+---------+    180
   N  V  N  L  E  E  K  I  D  V  V  P  I  E  P  H  A  L  F  L

   ggtatccacggtggtaaaatgtgcctgagctgcgtgaaatctggtgacgaaactcgtctg
181 ---------+---------+---------+---------+---------+---------+    240
   G  I  H  G  G  K  M  C  L  S  C  V  K  S  G  D  E  T  R  L

   cagctggaagcagttaacatcactgacctgagcgaaaaccgcaaacaggacaaacgtttc
241 ---------+---------+---------+---------+---------+---------+    300
   Q  L  E  A  V  N  I  T  D  L  S  E  N  R  K  Q  D  K  R  F

   gcattcatccgctctgacagcggcccgaccaccagcttcgaatctgctgcttgcccgggt
301 ---------+---------+---------+---------+---------+---------+    360
   A  F  I  R  S  D  S  G  P  T  T  S  F  E  S  A  A  C  P  G

   tggttcctgtgcactgctatggaagctgaccagccggtaagcctgaccaacatgccggac
361 ---------+---------+---------+---------+---------+---------+    420
   W  F  L  C  T  A  M  E  A  D  Q  P  V  S  L  T  N  M  P  D

            BstEII                                    HindIII
              |                                         |
   gaaggcgtgatggtaaccaaattctacttccaggaagacgaataatgggaagctt
421 ----------+--------+---------+---------+---------+-----     475
   E  G  V  M  V  T  K  F  Y  F  Q  E  D  E  *
```

The resulting plasmid pAMG21-IL-1ra was purified and the sequence of IL-1ra gene was confirmed by sequencing. This plasmid (pAMG21-IL-1ra), pAMG21-OPG-Fc and pAMG21-Fc-OPG (European Patent Application No. 96309363.8) were used later for cloning of RHUIL-1RA-FC protein. Two rhuIL-1ra Fc fusion proteins were constructed where the Fc region of human IgG1 was fused at either the N-terminus ("Fc-rhuIL-1ra") or the C-terminus ("rhuIL-1ra-Fc") of human IL-1ra. The Fc sequence that was chosen for fusions is shown below. Eight extra amino acid residues AAAEPKSS are present in the N-terminus of the functional Fc region.

Map of Fc3A C8S:

PstI

GCTGCAGCTGAACCAAAATCTTCCGACAAAACTCACACATGCCCACCGTGCCCAGCACCT (SEQ ID NO 5)

A A A E P K S S D K T H T C P P C P A P (SEQ ID NO 6)

The underlined sequence was added to the Fc region

GAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATG

E L L G G P S V F L F P P K P K D T L M

ATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAG

I S R T P E V T C V V V D V S H E D P E

SacII

GTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGG

V K F N W Y V D G V E V H N A K T K P R

GAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGAC

E E Q Y N S T Y R V V S V L T V L H Q D

TGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATC

W L N G K E Y K C K V S N K A L P A P I

GAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCC

E K T I S K A K G Q P R E P Q V Y T L P

CCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTC

P S R D E L T K N Q V S L T C L V K G F

TATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAG

Y P S D I A V E W E S N G Q P E N N Y K

ACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTG

T T P P V L D S D G S F F L Y S K L T V

GACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTG

D K S R W Q Q G N V F S C S V M H E A L

CACAACCACTACACGCAGAAGAGCCTCTCGCTCAGCCCGGGTAAA

H N H Y T Q K S L S L S P G K

B. Description of *E. coli* Host Strain

A derivative of *E. coli* W1485 (a K12 strain) was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. (CGSC strain #6159). The strain is prototrophic, contains no lambda prophage, and has been cured of the sex factor, F.

Subsequently, the CGSC strain #6159 has been altered by selecting for spontaneous resistance to four different phages isolated from phage outbreaks that occurred while conducting fermentation research. A first round of phage-resistant mutant isolation, conferring resistance simultaneously to two of the four phages, was performed. A sample of one of the phages was diluted and mixed with a culture of the sensitive strain and incubated as a liquid culture at 37° C. for 16–24 hours to select for phage-resistant survivors. Candidates were isolated from single colonies and tested to confirm phage resistance and ability to grow in minimal medium. The mutation obtained in the first round of selection exhibits characteristics of a tonA mutation in that the strain simultaneously acquired resistance to phages T5 and Φ80.

A second phage resistance selection, conferring resistance to the third phage, was performed on May 15, 1984. Spontaneous phage-resistant mutants were obtained using a plate method. Lawns of sensitive bacteria were spotted with a phage suspension and incubated at 37° C. for two days. Survivors were isolated from colonies in the zone of lysis. They were tested for growth in minimal medium, normal efficiency of plasmid transformation, normal growth rate in complex medium, and normal level of product synthesis. The mutation conferring resistance to this phage has been mapped at the btu locus of *E. coli*.

A third round of phage resistance selection was performed, using the plate method described above. The purified mutant appeared normal by those criteria outlined above (growth in minimal and complex media, efficiency of transformation and level of product synthesis).

C. Fc-rhuIL-1ra

The unique SacII site in the Fc region and the unique SacI site in the IL-1ra gene were used for cloning. The SacII-SacI fragment was synthesized using standard PCR technology.

Templates for PCR reactions were plasmid preparations (pAMG21-OPG-Fc and pAMG21-IL-1ra) containing the target genes. Overlapping oligos were designed to combine the C-terminal portion of Fc gene with the N terminal portion of the IL-1ra gene. This process allows fusing the two genes together in the correct reading frame after the appropriate PCR reactions have been performed. Initially, one "fusion" oligo for each gene, Oligo #1561-57 for Fc and #1561-56 for IL-1ra, was put into a PCR reaction with a primer 5' to the SacII in Fc (# 1561–55) or the SacI site in IL-1ra (#1561-58). At the end of this first PCR reaction, two separate products were obtained, with each individual gene having the fusion site present. In the second round of PCR, the first two PCR products were combined along with the two outside primers (# 1561-55 and #1561-58) and the full length fusion DNA sequence was produced.

The final PCR gene products were digested with restriction endonucleases SacII and SacI, and a three-way ligation was conducted with the ClaI-SacII Fc fragment with partial pAMG21 sequence isolated from pAMG21-Fc-OPG and the vector ClaI-SacI fragment with partial IL-1ra sequence isolated from pAMG21-IL-1ra. The ligation mixture was transformed into *E. coli* host by electroporation utilizing the manufacturer's protocol. Clones were screened for the ability to produce the recombinant Fc-rhuIL-1ra and to possess the gene fusion having the correct nucleotide sequence. A methionine residue was added to the junction of the Fc region and the rhuIL-1ra, but it did not interfere with the activity of the fusion protein.

The following primers were used to construct this Fc-rhuIL-1ra:

1651-55

5'-CCA CGA AGA CCC TGA GGT C-3' (SEQ ID NO 7)

1561-56

5'-GGG TAA AAT GCG ACC GTC CGG CCG TAA G-3' (SEQ ID NO 8)

1561-57

5'-GGA CGG TCG CAT TTT ACC CGG GCT GAG C-3' (SEQ ID NO 9)

1661-58

5'-CTG GTT GTT GCG CAG GTA G-3' (SEQ ID NO 10)

The following sequence of the open reading frame of complete Fc-rhuIL-1ra fusion gene is set forth in FIG. **11**.

D. rhuIL-1ra-Fc

Since the rhuIL-1ra-Fc fusion junction is flanked by an unique BstEII restriction site in IL-1ra and an PstI site in Fc, the BstEII-PstI fragment of about 35 base pairs was chemically synthesized instead of PCR synthesis. The upper strand (oligo #1561-52) and the lower strand (oligo #1561-53) were synthesized in a way to generate the cohesive ends of BstEII and PstI after they anneal to each other. A four-way ligation was conducted using the annealed BstEII-PstI chemically synthesized fragment, the PstI-BamHI enzyme digested Fc fragment from pAMG21-OPG-Fc, the BstEII-BstEII enzyme digested IL-1ra fragment with partial vector pAMG21 sequence from pAMG21-IL-1ra and the BstEII-BamHI enzyme digested vector pAMG21 fragment. The two BstEII sites in IL-1ra and pAMG21 do not share the same cohesive ends, therefore ligation in the wrong orientation was not a concern. The ligation mixture was transformed into *E. coli* host by electroporation utilizing the manufacturer's protocol. Clones were selected, plasmid DNA was isolated, and DNA sequencing was performed to verify the DNA sequence of the rhuIL-1ra-Fc fusion gene. The expression of rhuIL-1ra-Fc was detected on a Coomassie stained PAGE gel and on a Western blot.

The following primers were used to construct this rhuIL-1ra-Fc:

1561-52

5'-GTA ACC AAA TTC TAC TTC CAG GAA GAC GAA GCT GCA-3' (SEQ ID NO 11)

1561-53

5'-GCT TCG TCT TCC TGG AAG TAG AAT TTG-3' (SEQ ID NO 12)

The following sequence of the open reading frame of complete Fc-IL-1ra fusion gene is set forth in FIG. **12**.

E. Expression of IL-1ra-Fc Fusion Protein and Fc-IL-1ra Fusion Proteins in *E. coli*

A DNA sequence coding for IL-1ra-Fc fusion protein or an Fc-IL-1ra fusion protein was placed under control of the luxPR promoter in pAMG21 (U.S. Pat. No. No. 5,169,318 for description of the lux expression system).

Cultures of pAMG21-Fc-IL-1ra and pAMG21-IL-1ra-Fc in *E. coli* host were placed in Terrific broth media (Tartof. and Hobbs (1987), Bethesda Res. Lab. *Focus,* 9:12) containing 50 μg/ml kanamycin and were incubated at 30° C. to an OD600 of about 0.8 prior to induction. Induction of recombinant gene product expression from the luxPR promoter of vector pAMG21 was achieved following the addition of the synthetic autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture media to a final concentration of 30 ng/ml and incubation at 37° C. for a further 6 hours. After 6 hours, bacterial cultures were pelleted by centrifugation. The pelleted cultures were resuspended, lysed by sonication, and soluble and insoluble fractions were separated by centrifugation. The whole cell lysate, and the soluble and insoluble fractions were analyzed by SDS-polyacrylamide gel electrophoresis and by Western blot. The induced cultures at 37° C. have inclusion bodies, and over 70% of the product is in the insoluble fraction.

F. Purification of IL-1ra-Fc Fusion Protein

Cells were broken by high pressure homogenization (2 passes at 14,000 psi in a microfluidizer (Microfluidics Corp., Newton, Mass. and the inclusion bodies were harvested by centrifugation at 4200 RPM in a J-6B™ centrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The inclusion bodies were solubilized at a 1 to 10 (w/v) in 6M guanidine-HCl, 50 mM tris, 7 mM DTT, pH 8.7 for one hour. The solubilized inclusion bodies were diluted 20 fold into 1.5M urea, 40 mM tris, 500 mM arginine, 4 mM cysteine, 1 mM cystamine dihydrochloride, pH 8.5 and stirred in the cold room. After about one day the mixture was concentrated about tenfold and buffer exchanged into 20 mM tris, 100 mM arginine, 800 mM urea, pH 8.5 using a pellicon ultrafiltration device in the cold. This mixture was adjusted to pH 5 with acetic acid and the precipitated material was centrifuged away. The supernatant was applied to an SP-Sepharose™ column (Pharmacia Biotech, Inc., Piscataway, N.J. equilibrated in 20 mM sodium acetate, 100 mM arginine, pH 5 in the cold. After loading the column was washed with the same buffer. The IL-1ra Fc was eluted using a 20 column volume gradient from 0 to 500 mM NaCl in equilibration buffer. Peak fractions were pooled after SDS-PAGE analysis. To the pool was added sodium phoshate to

10 mM and the pH adjusted to 7. Ammonium sulfate was then added to 700 mM and the sample was applied to a Phenyl Toyopearl column (Toso Haas, Philadelphia, Pa.) equilibrated in 10 mM sodium phosphate, 700 mM ammonium sulfate, pH 7 at room temperature. After loading the column was washed with the same buffer and the Il-1ra-Fc fusion protein was eluted using a 20 column volume gradient from 700 mM to 0 mM ammonium sulfate in 10 mM sodium phosphate, pH 7. Peak fractions were pooled after SDS-PAGE analysis and the pool was concentrated about 4 fold and buffer exchanged using a Minisette™ ultrafiltration device (Filtron, Northborough, Mass.) into 10 mM sodium phosphate, pH 6.7. This sample was then applied to an SP Sepharose HP™ column (Pharmacia Biotech, Inc., Piscataway, N.J. equilibrated in 10 mM sodium phosphate, pH 6.7 in the cold. After loading and washing the column, the IL-1ra-Fc was eluted using a 20 column volume gradient from 0 to 150 mM NaCl in equilibration buffer. The peak was pooled and filtered.

While the present invention has been described above both generally and in terms of preferred embodiments, it is understood that other variations and modifications will occur to those skilled in the art in light of the description above.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 16

<210> SEQ ID NO 1
<211> LENGTH: 462
<212> TYPE: DNA
<213> ORGANISM: Human
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(462)
<223> OTHER INFORMATION: Initial methionine is optional

<400> SEQUENCE: 1

```
atg cga ccc tct ggg aga aaa tcc agc aag atg caa gcc ttc aga atc       48
Met Arg Pro Ser Gly Arg Lys Ser Ser Lys Met Gln Ala Phe Arg Ile
  1               5                  10                  15

tgg gat gtt aac cag aag acc ttc tat ctg agg aac aac caa cta gtt       96
Trp Asp Val Asn Gln Lys Thr Phe Tyr Leu Arg Asn Asn Gln Leu Val
             20                  25                  30

gct gga tac ttg caa gga cca aat gtc aat tta gaa gaa aag ata gat      144
Ala Gly Tyr Leu Gln Gly Pro Asn Val Asn Leu Glu Glu Lys Ile Asp
         35                  40                  45

gtg gta ccc att gag cct cat gct ctg ttc ttg gga atc cat gga ggg      192
Val Val Pro Ile Glu Pro His Ala Leu Phe Leu Gly Ile His Gly Gly
     50                  55                  60

aag atg tgc ctg tcc tgt gtc aag tct ggt gat gag acc aga ctc cag      240
Lys Met Cys Leu Ser Cys Val Lys Ser Gly Asp Glu Thr Arg Leu Gln
 65                  70                  75                  80

ctg gag gca gtt aac atc act gac ctg agc gag aac aga aag cag gac      288
Leu Glu Ala Val Asn Ile Thr Asp Leu Ser Glu Asn Arg Lys Gln Asp
                 85                  90                  95

aag cgc ttc gcc ttc atc cgc tca gac agt ggc ccc acc acc agt ttt      336
Lys Arg Phe Ala Phe Ile Arg Ser Asp Ser Gly Pro Thr Thr Ser Phe
            100                 105                 110

gag tct gcc gcc tgc ccc ggt tgg ttc ctc tgc aca gcg atg gaa gct      384
Glu Ser Ala Ala Cys Pro Gly Trp Phe Leu Cys Thr Ala Met Glu Ala
        115                 120                 125

gac cag ccc gtc agc ctc acc aat atg cct gac gaa ggc gtc atg gtc      432
Asp Gln Pro Val Ser Leu Thr Asn Met Pro Asp Glu Gly Val Met Val
    130                 135                 140

acc aaa ttc tac ttc cag gag gac gag tag                              462
Thr Lys Phe Tyr Phe Gln Glu Asp Glu
145                 150
```

<210> SEQ ID NO 2
<211> LENGTH: 153
<212> TYPE: PRT
<213> ORGANISM: Human

-continued

<400> SEQUENCE: 2

```
Met Arg Pro Ser Gly Arg Lys Ser Ser Lys Met Gln Ala Phe Arg Ile
  1               5                  10                  15

Trp Asp Val Asn Gln Lys Thr Phe Tyr Leu Arg Asn Asn Gln Leu Val
            20                  25                  30

Ala Gly Tyr Leu Gln Gly Pro Asn Val Asn Leu Glu Glu Lys Ile Asp
        35                  40                  45

Val Val Pro Ile Glu Pro His Ala Leu Phe Leu Gly Ile His Gly Gly
    50                  55                  60

Lys Met Cys Leu Ser Cys Val Lys Ser Gly Asp Glu Thr Arg Leu Gln
65                  70                  75                  80

Leu Glu Ala Val Asn Ile Thr Asp Leu Ser Glu Asn Arg Lys Gln Asp
                85                  90                  95

Lys Arg Phe Ala Phe Ile Arg Ser Asp Ser Gly Pro Thr Thr Ser Phe
            100                 105                 110

Glu Ser Ala Ala Cys Pro Gly Trp Phe Leu Cys Thr Ala Met Glu Ala
        115                 120                 125

Asp Gln Pro Val Ser Leu Thr Asn Met Pro Asp Glu Gly Val Met Val
    130                 135                 140

Thr Lys Phe Tyr Phe Gln Glu Asp Glu
145                 150
```

<210> SEQ ID NO 3
<211> LENGTH: 475
<212> TYPE: DNA
<213> ORGANISM: Human
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (4)..(465)

<400> SEQUENCE: 3

```
cat atg cga ccg tcc ggc cgt aag agc tcc aaa atg cag gct ttc cgt      48
    Met Arg Pro Ser Gly Arg Lys Ser Ser Lys Met Gln Ala Phe Arg
      1               5                  10                  15

atc tgg gac gtt aac cag aaa acc ttc tac ctg cgc aac aac cag ctg      96
Ile Trp Asp Val Asn Gln Lys Thr Phe Tyr Leu Arg Asn Asn Gln Leu
                20                  25                  30

gtt gct ggc tac ctg cag ggt ccg aac gtt aac ctg gaa gaa aaa atc     144
Val Ala Gly Tyr Leu Gln Gly Pro Asn Val Asn Leu Glu Glu Lys Ile
            35                  40                  45

gac gtt gta ccg atc gaa ccg cac gct ctg ttc ctg ggt atc cac ggt     192
Asp Val Val Pro Ile Glu Pro His Ala Leu Phe Leu Gly Ile His Gly
        50                  55                  60

ggt aaa atg tgc ctg agc tgc gtg aaa tct ggt gac gaa act cgt ctg     240
Gly Lys Met Cys Leu Ser Cys Val Lys Ser Gly Asp Glu Thr Arg Leu
    65                  70                  75

cag ctg gaa gca gtt aac atc act gac ctg agc gaa aac cgc aaa cag     288
Gln Leu Glu Ala Val Asn Ile Thr Asp Leu Ser Glu Asn Arg Lys Gln
80                  85                  90                  95

gac aaa cgt ttc gca ttc atc cgc tct gac agc ggc ccg acc acc agc     336
Asp Lys Arg Phe Ala Phe Ile Arg Ser Asp Ser Gly Pro Thr Thr Ser
                100                 105                 110

ttc gaa tct gct gct tgc ccg ggt tgg ttc ctg tgc act gct atg gaa     384
Phe Glu Ser Ala Ala Cys Pro Gly Trp Phe Leu Cys Thr Ala Met Glu
            115                 120                 125

gct gac cag ccg gta agc ctg acc aac atg ccg gac gaa ggc gtg atg     432
Ala Asp Gln Pro Val Ser Leu Thr Asn Met Pro Asp Glu Gly Val Met
        130                 135                 140
```

-continued

```
gta acc aaa ttc tac ttc cag gaa gac gaa taa tgggaagctt              475
Val Thr Lys Phe Tyr Phe Gln Glu Asp Glu
    145                 150
```

<210> SEQ ID NO 4
<211> LENGTH: 153
<212> TYPE: PRT
<213> ORGANISM: Human

<400> SEQUENCE: 4

```
Met Arg Pro Ser Gly Arg Lys Ser Ser Lys Met Gln Ala Phe Arg Ile
  1               5                  10                  15

Trp Asp Val Asn Gln Lys Thr Phe Tyr Leu Arg Asn Asn Gln Leu Val
             20                  25                  30

Ala Gly Tyr Leu Gln Gly Pro Asn Val Asn Leu Glu Glu Lys Ile Asp
         35                  40                  45

Val Val Pro Ile Glu Pro His Ala Leu Phe Leu Gly Ile His Gly Gly
     50                  55                  60

Lys Met Cys Leu Ser Cys Val Lys Ser Gly Asp Glu Thr Arg Leu Gln
 65                  70                  75                  80

Leu Glu Ala Val Asn Ile Thr Asp Leu Ser Glu Asn Arg Lys Gln Asp
                 85                  90                  95

Lys Arg Phe Ala Phe Ile Arg Ser Asp Ser Gly Pro Thr Thr Ser Phe
            100                 105                 110

Glu Ser Ala Ala Cys Pro Gly Trp Phe Leu Cys Thr Ala Met Glu Ala
        115                 120                 125

Asp Gln Pro Val Ser Leu Thr Asn Met Pro Asp Glu Gly Val Met Val
    130                 135                 140

Thr Lys Phe Tyr Phe Gln Glu Asp Glu
145                 150
```

<210> SEQ ID NO 5
<211> LENGTH: 705
<212> TYPE: DNA
<213> ORGANISM: Human
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(705)

<400> SEQUENCE: 5

```
gct gca gct gaa cca aaa tct tcc gac aaa act cac aca tgc cca ccg       48
Ala Ala Ala Glu Pro Lys Ser Ser Asp Lys Thr His Thr Cys Pro Pro
  1               5                  10                  15

tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc       96
Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro
             20                  25                  30

cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc aca      144
Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr
         35                  40                  45

tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc aac      192
Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn
     50                  55                  60

tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg      240
Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg
 65                  70                  75                  80

gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc      288
Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
                 85                  90                  95

ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc      336
Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser
```

-continued

```
            100                 105                 110

aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa      384
Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys
        115                 120                 125

ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg gat      432
Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp
    130                 135                 140

gag ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc      480
Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
145                 150                 155                 160

tat ccc agc gac atc gcc gtg gag tgg gag agc aat ggg cag ccg gag      528
Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
                165                 170                 175

aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc      576
Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe
            180                 185                 190

ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag cag ggg      624
Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly
        195                 200                 205

aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac tac      672
Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr
    210                 215                 220

acg cag aag agc ctc tcg ctc agc ccg ggt aaa                         705
Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
225                 230                 235


<210> SEQ ID NO 6
<211> LENGTH: 235
<212> TYPE: PRT
<213> ORGANISM: Human

<400> SEQUENCE: 6

Ala Ala Ala Glu Pro Lys Ser Ser Asp Lys Thr His Thr Cys Pro Pro
  1               5                  10                  15

Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro
             20                  25                  30

Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr
         35                  40                  45

Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn
     50                  55                  60

Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg
 65                  70                  75                  80

Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
                 85                  90                  95

Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser
            100                 105                 110

Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys
        115                 120                 125

Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp
    130                 135                 140

Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
145                 150                 155                 160

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
                165                 170                 175

Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe
            180                 185                 190

Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly
```

-continued

195 200 205

Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr
210 215 220

Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
225 230 235

<210> SEQ ID NO 7
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Human

<400> SEQUENCE: 7

ccacgaagac cctgaggtc 19

<210> SEQ ID NO 8
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Human

<400> SEQUENCE: 8

gggtaaaatg cgaccgtccg gccgtaag 28

<210> SEQ ID NO 9
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Human

<400> SEQUENCE: 9

ggacggtcgc attttacccg ggctgagc 28

<210> SEQ ID NO 10
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Human

<400> SEQUENCE: 10

ctggttgttg cgcaggtag 19

<210> SEQ ID NO 11
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Human

<400> SEQUENCE: 11

gtaaccaaat tctacttcca ggaagacgaa gctgca 36

<210> SEQ ID NO 12
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Human

<400> SEQUENCE: 12

gcttcgtctt cctggaagta gaatttg 27

<210> SEQ ID NO 13
<211> LENGTH: 1170
<212> TYPE: DNA
<213> ORGANISM: Human
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(1170)

<400> SEQUENCE: 13

-continued

```
atg gct gca gct gaa cca aaa tct tcc gac aaa act cac aca tgc cca      48
Met Ala Ala Ala Glu Pro Lys Ser Ser Asp Lys Thr His Thr Cys Pro
  1               5                  10                  15

ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc      96
Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe
             20                  25                  30

ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc     144
Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val
         35                  40                  45

aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc     192
Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe
     50                  55                  60

aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg     240
Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro
 65                  70                  75                  80

cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc     288
Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr
                 85                  90                  95

gtc ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc     336
Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
            100                 105                 110

tcc aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc     384
Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala
        115                 120                 125

aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg     432
Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg
    130                 135                 140

gat gag ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc     480
Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly
145                 150                 155                 160

ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat ggg cag ccg     528
Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
                165                 170                 175

gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc     576
Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
            180                 185                 190

ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag cag     624
Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln
        195                 200                 205

ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac     672
Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His
    210                 215                 220

tac acg cag aag agc ctc tcg ctc agc ccg ggt aaa atg cga ccg tcc     720
Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Met Arg Pro Ser
225                 230                 235                 240

ggc cgt aag agc tcc aaa atg cag gct ttc cgt atc tgg gac gtt aac     768
Gly Arg Lys Ser Ser Lys Met Gln Ala Phe Arg Ile Trp Asp Val Asn
                245                 250                 255

cag aaa acc ttc tac ctg cgc aac aac cag ctg gtt gct ggc tac ctg     816
Gln Lys Thr Phe Tyr Leu Arg Asn Asn Gln Leu Val Ala Gly Tyr Leu
            260                 265                 270

cag ggt ccg aac gtt aac ctg gaa gaa aaa atc gac gtt gta ccg atc     864
Gln Gly Pro Asn Val Asn Leu Glu Glu Lys Ile Asp Val Val Pro Ile
        275                 280                 285

gaa ccg cac gct ctg ttc ctg ggt atc cac ggt ggt aaa atg tgc ctg     912
Glu Pro His Ala Leu Phe Leu Gly Ile His Gly Gly Lys Met Cys Leu
    290                 295                 300

agc tgc gtg aaa tct ggt gac gaa act cgt ctg cag ctg gaa gca gtt     960
Ser Cys Val Lys Ser Gly Asp Glu Thr Arg Leu Gln Leu Glu Ala Val
305                 310                 315                 320
```

-continued

```
aac atc act gac ctg agc gaa aac cgc aaa cag gac aaa cgt ttc gca     1008
Asn Ile Thr Asp Leu Ser Glu Asn Arg Lys Gln Asp Lys Arg Phe Ala
                325                 330                 335

ttc atc cgc tct gac agc ggc ccg acc acc agc ttc gaa tct gct gct     1056
Phe Ile Arg Ser Asp Ser Gly Pro Thr Thr Ser Phe Glu Ser Ala Ala
            340                 345                 350

tgc ccg ggt tgg ttc ctg tgc act gct atg gaa gct gac cag ccg gta     1104
Cys Pro Gly Trp Phe Leu Cys Thr Ala Met Glu Ala Asp Gln Pro Val
        355                 360                 365

agc ctg acc aac atg ccg gac gaa ggc gtg atg gta acc aaa ttc tac     1152
Ser Leu Thr Asn Met Pro Asp Glu Gly Val Met Val Thr Lys Phe Tyr
    370                 375                 380

ttc cag gaa gac gaa taa                                            1170
Phe Gln Glu Asp Glu
385                 390
```

<210> SEQ ID NO 14
<211> LENGTH: 389
<212> TYPE: PRT
<213> ORGANISM: Human

<400> SEQUENCE: 14

```
Met Ala Ala Ala Glu Pro Lys Ser Ser Asp Lys Thr His Thr Cys Pro
  1               5                  10                  15

Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe
             20                  25                  30

Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val
         35                  40                  45

Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe
     50                  55                  60

Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro
 65                  70                  75                  80

Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr
                 85                  90                  95

Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
            100                 105                 110

Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala
        115                 120                 125

Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg
    130                 135                 140

Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly
145                 150                 155                 160

Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
                165                 170                 175

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
            180                 185                 190

Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln
        195                 200                 205

Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His
    210                 215                 220

Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Met Arg Pro Ser
225                 230                 235                 240

Gly Arg Lys Ser Ser Lys Met Gln Ala Phe Arg Ile Trp Asp Val Asn
                245                 250                 255

Gln Lys Thr Phe Tyr Leu Arg Asn Asn Gln Leu Val Ala Gly Tyr Leu
            260                 265                 270
```

-continued

```
Gln Gly Pro Asn Val Asn Leu Glu Glu Lys Ile Asp Val Val Pro Ile
        275                 280                 285

Glu Pro His Ala Leu Phe Leu Gly Ile His Gly Gly Lys Met Cys Leu
    290                 295                 300

Ser Cys Val Lys Ser Gly Asp Glu Thr Arg Leu Gln Leu Glu Ala Val
305                 310                 315                 320

Asn Ile Thr Asp Leu Ser Glu Asn Arg Lys Gln Asp Lys Arg Phe Ala
                325                 330                 335

Phe Ile Arg Ser Asp Ser Gly Pro Thr Thr Ser Phe Glu Ser Ala Ala
            340                 345                 350

Cys Pro Gly Trp Phe Leu Cys Thr Ala Met Glu Ala Asp Gln Pro Val
        355                 360                 365

Ser Leu Thr Asn Met Pro Asp Glu Gly Val Met Val Thr Lys Phe Tyr
    370                 375                 380

Phe Gln Glu Asp Glu
385
```

<210> SEQ ID NO 15
<211> LENGTH: 1167
<212> TYPE: DNA
<213> ORGANISM: Human
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)..(1167)

<400> SEQUENCE: 15

```
atg cga ccg tcc ggc cgt aag agc tcc aaa atg cag gct ttc cgt atc      48
Met Arg Pro Ser Gly Arg Lys Ser Ser Lys Met Gln Ala Phe Arg Ile
  1               5                  10                  15

tgg gac gtt aac cag aaa acc ttc tac ctg cgc aac aac cag ctg gtt      96
Trp Asp Val Asn Gln Lys Thr Phe Tyr Leu Arg Asn Asn Gln Leu Val
            20                  25                  30

gct ggc tac ctg cag ggt ccg aac gtt aac ctg gaa gaa aaa atc gac     144
Ala Gly Tyr Leu Gln Gly Pro Asn Val Asn Leu Glu Glu Lys Ile Asp
        35                  40                  45

gtt gta ccg atc gaa ccg cac gct ctg ttc ctg ggt atc cac ggt ggt     192
Val Val Pro Ile Glu Pro His Ala Leu Phe Leu Gly Ile His Gly Gly
    50                  55                  60

aaa atg tgc ctg agc tgc gtg aaa tct ggt gac gaa act cgt ctg cag     240
Lys Met Cys Leu Ser Cys Val Lys Ser Gly Asp Glu Thr Arg Leu Gln
65                  70                  75                  80

ctg gaa gca gtt aac atc act gac ctg agc gaa aac cgc aaa cag gac     288
Leu Glu Ala Val Asn Ile Thr Asp Leu Ser Glu Asn Arg Lys Gln Asp
                85                  90                  95

aaa cgt ttc gca ttc atc cgc tct gac agc ggc ccg acc acc agc ttc     336
Lys Arg Phe Ala Phe Ile Arg Ser Asp Ser Gly Pro Thr Thr Ser Phe
            100                 105                 110

gaa tct gct gct tgc ccg ggt tgg ttc ctg tgc act gct atg gaa gct     384
Glu Ser Ala Ala Cys Pro Gly Trp Phe Leu Cys Thr Ala Met Glu Ala
        115                 120                 125

gac cag ccg gta agc ctg acc aac atg ccg gac gaa ggc gtg atg gta     432
Asp Gln Pro Val Ser Leu Thr Asn Met Pro Asp Glu Gly Val Met Val
    130                 135                 140

acc aaa ttc tac ttc cag gaa gac gaa gct gca gct gaa cca aaa tct     480
Thr Lys Phe Tyr Phe Gln Glu Asp Glu Ala Ala Ala Glu Pro Lys Ser
145                 150                 155                 160

tcc gac aaa act cac aca tgc cca ccg tgc cca gca cct gaa ctc ctg     528
Ser Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
                165                 170                 175
```

-continued

```
ggg gga ccg tca gtc ttc ctc ttc ccc cca aaa ccc aag gac acc ctc      576
Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
            180                 185                 190

atg atc tcc cgg acc cct gag gtc aca tgc gtg gtg gtg gac gtg agc      624
Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
        195                 200                 205

cac gaa gac cct gag gtc aag ttc aac tgg tac gtg gac ggc gtg gag      672
His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
    210                 215                 220

gtg cat aat gcc aag aca aag ccg cgg gag gag cag tac aac agc acg      720
Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
225                 230                 235                 240

tac cgt gtg gtc agc gtc ctc acc gtc ctg cac cag gac tgg ctg aat      768
Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
                245                 250                 255

ggc aag gag tac aag tgc aag gtc tcc aac aaa gcc ctc cca gcc ccc      816
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
            260                 265                 270

atc gag aaa acc atc tcc aaa gcc aaa ggg cag ccc cga gaa cca cag      864
Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
        275                 280                 285

gtg tac acc ctg ccc cca tcc cgg gat gag ctg acc aag aac cag gtc      912
Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val
    290                 295                 300

agc ctg acc tgc ctg gtc aaa ggc ttc tat ccc agc gac atc gcc gtg      960
Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
305                 310                 315                 320

gag tgg gag agc aat ggg cag ccg gag aac aac tac aag acc acg cct     1008
Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
                325                 330                 335

ccc gtg ctg gac tcc gac ggc tcc ttc ttc ctc tac agc aag ctc acc     1056
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
            340                 345                 350

gtg gac aag agc agg tgg cag cag ggg aac gtc ttc tca tgc tcc gtg     1104
Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
        355                 360                 365

atg cat gag gct ctg cac aac cac tac acg cag aag agc ctc tcg ctc     1152
Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
    370                 375                 380

agc ccg ggt aaa taa                                             1167
Ser Pro Gly Lys
385
```

<210> SEQ ID NO 16
<211> LENGTH: 388
<212> TYPE: PRT
<213> ORGANISM: Human

<400> SEQUENCE: 16

```
Met Arg Pro Ser Gly Arg Lys Ser Ser Lys Met Gln Ala Phe Arg Ile
  1               5                  10                  15

Trp Asp Val Asn Gln Lys Thr Phe Tyr Leu Arg Asn Asn Gln Leu Val
            20                  25                  30

Ala Gly Tyr Leu Gln Gly Pro Asn Val Asn Leu Glu Glu Lys Ile Asp
        35                  40                  45

Val Val Pro Ile Glu Pro His Ala Leu Phe Leu Gly Ile His Gly Gly
    50                  55                  60

Lys Met Cys Leu Ser Cys Val Lys Ser Gly Asp Glu Thr Arg Leu Gln
65                  70                  75                  80
```

-continued

```
Leu Glu Ala Val Asn Ile Thr Asp Leu Ser Glu Asn Arg Lys Gln Asp
                85                  90                  95

Lys Arg Phe Ala Phe Ile Arg Ser Asp Ser Gly Pro Thr Thr Ser Phe
            100                 105                 110

Glu Ser Ala Ala Cys Pro Gly Trp Phe Leu Cys Thr Ala Met Glu Ala
        115                 120                 125

Asp Gln Pro Val Ser Leu Thr Asn Met Pro Asp Glu Gly Val Met Val
    130                 135                 140

Thr Lys Phe Tyr Phe Gln Glu Asp Glu Ala Ala Ala Glu Pro Lys Ser
145                 150                 155                 160

Ser Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
                165                 170                 175

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
            180                 185                 190

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
        195                 200                 205

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
    210                 215                 220

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
225                 230                 235                 240

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
                245                 250                 255

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
            260                 265                 270

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
        275                 280                 285

Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val
    290                 295                 300

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
305                 310                 315                 320

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
                325                 330                 335

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
            340                 345                 350

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
        355                 360                 365

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
    370                 375                 380

Ser Pro Gly Lys
385
```

What is claimed is:

1. A fusion protein comprising an interleukin-1 receptor antagonist (IL-1ra) which comprises:

(a) the amino acid sequence of SEQ ID NO: 2, or

(b) an IL-1 inhibitory fragment of the amino acid sequence of SEQ ID NO:2 in which 1 to 30 amino acids are deleted from the N-terminus or C-terminus, or

(c) an IL-1 inhibitory sequence which is at least 70% homologous to the amino acid sequence of SEQ ID NO: 2;

with a constant domain of a heavy or light chain of human immunoglobulin at the amino-terminus of said IL-1ra.

2. The fusion protein according to claim **1**, wherein said IL-1ra is non-glycosylated.

3. The fusion protein according to claim **2**, wherein said IL-1ra is methionyl IL-1ra.

4. The fusion protein according to claim **1**, wherein said IL-1ra is glycosylated.

5. The fusion protein according to claim **1**, wherein said human immunoglobulin is the constant domain of a heavy chain.

6. The fusion protein according to claim **5**, wherein said heavy chain is IgG1.

7. The fusion protein according to claim **1**, wherein said constant domain comprises all domains except the first domain of the constant region of such heavy chain of human immunoglobulin.

8. The fusion protein according to claim **7**, wherein said heavy chain is selected from the group consisting of IgG, IgA, IgM or IgE.

9. The fusion protein according to claim **8**, wherein said IgG is IgG1 or IgG3.

10. The fusion protein according to claim **1**, wherein said human immunoglobulin is the constant domain of a heavy chain.

11. The fusion protein according to claim **10**, wherein said heavy chain is IgG1.

12. The fusion protein according to claim **1**, wherein said constant domain comprises all domains except the first domain of the constant region of such heavy chain of human immunoglobulin.

13. The fusion protein according to claim **12**, wherein said heavy chain is selected from the group consisting of IgG, IgA, IgM or IgE.

14. The fusion protein according to claim **13**, wherein said IgG is IgG1 or IgG3.

15. A pharmaceutical composition comprising an effective amount of a fusion protein according to any one of claims **1** and **2–14** and a pharmaceutically acceptable carrier.

* * * * *

US006159460A

United States Patent [19]

Thompson et al.

[11] Patent Number: 6,159,460

[45] Date of Patent: Dec. 12, 2000

[54] **METHOD FOR TREATING INTERLEUKIN-1 MEDIATED DISEASES**

[75] Inventors: **Robert C. Thompson**; **David F. Carmichael**, both of Boulder, Colo.

[73] Assignee: **Amgen Inc.**, Thousand Oaks, Calif.

[21] Appl. No.: **08/292,539**

[22] Filed: **Aug. 18, 1994**

Related U.S. Application Data

[63] Continuation-in-part of application No. 08/002,074, Jan. 8, 1993, abandoned, and a continuation-in-part of application No. 08/171,873, Dec. 22, 1993, abandoned, and a continuation-in-part of application No. 08/171,876, Dec. 22, 1993, abandoned, and a continuation-in-part of application No. 08/171,867, Dec. 22, 1993, abandoned, said application No. 08/002,074, is a continuation-in-part of application No. 07/849,635, Mar. 5, 1992, abandoned, which is a continuation of application No. 08/502,745, Apr. 2, 1990, abandoned, which is a continuation-in-part of application No. 07/463,888, Jan. 11, 1990, abandoned, said application No. 08/171,873, is a continuation of application No. 08/047,765, Apr. 15, 1993, abandoned, which is a continuation of application No. 07/895,145, Jun. 5, 1992, abandoned, which is a continuation of application No. 07/524,210, May 16, 1990, abandoned, which is a continuation-in-part of application No. 07/502,745, Apr. 2, 1990, abandoned, said application No. 08/171,876, is a continuation of application No. 08/047,762, Apr. 15, 1993, abandoned, which is a continuation of application No. 07/895,153, Jun. 5, 1992, abandoned, which is a continuation of application No. 07/530,553, May 29, 1990, abandoned, which is a continuation-in-part of application No. 07/524,210, May 16, 1990, abandoned, said application No. 08/171,867, is a continuation of application No. 08/097,308, Jul. 26, 1993, abandoned, which is a continuation of application No. 07/936,874, Aug. 27, 1992, abandoned, which is a continuation of application No. 07/678,732, Apr. 1, 1991, abandoned, which is a continuation-in-part of application No. 07/530,553, May 29, 1990, abandoned.

[51] **Int. Cl.**[7] **A61K 38/19**

[52] **U.S. Cl.** **424/85.1**; 514/2; 514/8; 514/12; 514/885

[58] **Field of Search** 514/8, 12, 21, 514/885; 530/350; 424/85.1

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,935,343	6/1990	Allison et al.	435/7
4,956,381	9/1990	Bollinger et al.	514/443
4,968,607	11/1990	Dower et al.	435/69.1
5,041,554	8/1991	Parker et al.	548/532
5,075,222	12/1991	Hannum et al.	435/69.1
5,359,032	10/1994	Dayer et al.	530/350

FOREIGN PATENT DOCUMENTS

0 398 817 A1	11/1990	European Pat. Off. .
0 343 684 B1	4/1993	European Pat. Off. .
0 541 920 A1	5/1993	European Pat. Off. .
27 06772	12/1994	France .
2-223597	9/1990	Japan .
WO 89/01946	3/1989	WIPO .
WO 89/11540	11/1989	WIPO .
WO 91/00742	1/1991	WIPO .
WO 91/08285	6/1991	WIPO .
WO 91/17184	11/1991	WIPO .
WO 91/17249	11/1991	WIPO .
WO 92/12724	8/1992	WIPO .
WO 92/16221	10/1992	WIPO .
WO 93/02692	2/1993	WIPO .
WO 93/08304	4/1993	WIPO .
WO 93/08820	5/1993	WIPO .
WO 93/18783	9/1993	WIPO .
WO 93/21946	11/1993	WIPO .
WO 93/24134	12/1993	WIPO .
WO 94/06457	3/1994	WIPO .
WO 94/20517	9/1994	WIPO .
WO 94/21235	9/1994	WIPO .
WO 94/21275	9/1994	WIPO .
WO 95/10298	4/1995	WIPO .
WO 95/16353	6/1995	WIPO .
WO 95/16706	6/1995	WIPO .
WO 96/09323	3/1996	WIPO .

OTHER PUBLICATIONS

Bowie et al. Science 1990, vol. 247, pp. 1306–1310.

Tan et al. Australian & New Zealand Rhematism Associations. p. 113 (1986).

Biotechnology Bulletin, Jun. 31, 1994, vol. 13, No. 6, p. 2.

Bulletin International d'Informations (Droit et Pharmacie) Sep. 21, 1994, Aug. 9, p. 89.

Seckinger et al. (1987) J. of Immunol. vol. 139. pp. 1546–1549.

Seckinger et al. (1987) Ann. Inst. Pasteur Immunol. vol. 138. pp. 486–488.

Okusawa et al. (1988) J. Clin. Invest. vol. 81. pp. 1162–1172.

Sullivan et al. (1988) Infection & Immunity vol. 56 pp. 1722–1729.

Cominelli et al. (1989) Biotherapy vol. 1, pp. 369–375.

Dinarello et al. (1988) Digestive Diseases & Sciences vol. 33, pp. 255–355.

Billingham (1985) Br. J. of Rhematology vol. 24, pp. 25–28.

Pujol et al. (1987) Life Sciences vol. 41 pp. 1187–1198.

Relton & Rothwell , *Brain Res. Bull.* 29:243–246 (1992).

Rothwell & Relton, *Neurosci. Biobehav. Rev.* 17:217–227 (1993).

Conti et al, *Int. J. Immunopharmae* 14(6):987–993 (1992).

Seckinger et al, *J. Immunol.* 139:1546–1549 (1987).

Pujol & Loyau, *Life Sciences* 41:1187–1198 (1987).

Eisenberg et al, *Nature* 343:341–346 (1990).

Tan et al, *Australian & New Zealand Rheumatism Assoc. 1987* 17(1):113 (Abstract).

Seckinger & Dayer, *18th Forum in Immunol.* 486–488 (1987).

(List continued on next page.)

Primary Examiner—Prema Mertz

Attorney, Agent, or Firm—Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.

[57] **ABSTRACT**

The present invention provides methods for treating interleukin-1 mediated diseases including arthritis, inflammatory bowel disease, sepsis and septic shock, ischemia injury, reperfusion injury, multiple sclerosis and cerebral infarctions such as cerebral palsy. The methods are accomplished by administering to a patient in need thereof a therapeutically effective amount of an interleukin-1 inhibitor.

19 Claims, 4 Drawing Sheets

OTHER PUBLICATIONS

Billingham, *Br. J. Rheumatology* 24 (Suppl. 1):25–28 (1985).
Stimpson et al. *J. Immunol.* 140:2964–2969 (1988).
Hannum et al, *Nature* 343:336–340 (1990).
Arend et al, *J. Immunol.* 143(6): 1851–1858 (1989).
Cominelli et al, *Biotherapy* 1(4):369–375 (1989).
Cominelli et al, *Gastroenterology* 97(6): 1400–1405 (1989).
Dinarello, *Digestive Diseases & Sciences* 33(Suppl 3): 255–355 (1988).
Okusawa et al, *J. Clin. Invest.* 81(4): 1162–1172 (1988).
Sullivan et al, *Infect. Immunol.* 56(7): 1722–1729 (1988).
Dinarello, *Blood* 77(8): 1627–1652 (1991).
Cominelli et al, *J. Clin. Invest.* 86:972–980 (1990).
Ohlsson et al. *Nature* 348:550–552 (1990).
Ulich et al, *Am J. Pathology* 138(3):521–524 (1991).
Thomas et al, *Agents & Action* 34:187–190 (1991).
Seckinger et al, *J. Immunol.* 145:4181–4184 (1990).
Wakabayashi et al, *FASEB J.* 5(3):338–343 (1991).
Lotz et al, *Arthritis Rheum,* 29:S38 #162 (Abstract).
Poli et al, *Proc. Natl. Acad. Sci. USA* 91:108–112(1994).
Piguet et al, *Cytokine* 5(1): 57–61 (1993).
Wooley et al, *Arthritis & Rheumatism* 36(9): 1305–1314 (1993).
Rolfe et al, *Am. Rev. Respir. Dis.* 148:1378–1384 (1993).
Moldawer, *Blood Purif.* 11:128–133 (1993).
Pillay et al, *Pflugers Arch.* 424:549–551 (1993).
Ferrara, Keystone Symposium on Cellular Immunity & the Immuno–therapy of Cancer, Mar. 17–24, 1993, p. 96 (Abstract No. NZ 019).
Catalano, Keystone Symposium on Cytokines and Cytokine Receptors, Jan. 31–Feb. 7, 1993, p. 55 (Abstract No. E 016).

Cannon et al., "Circulating Interleukin–1 and Tumor Necrosis Factor in Septic Shock and Experimental Endotoxin Fever", *Journal of Infectious Diseases* 161:79–84 (1990).

Eichacker et al., "The Effects of Human Recombinant Interleukin–1 (IL–1) On Canine Alveolar Neutrophil (N) Number And Lung Function", *Critical Care Medicine,* Apr., 1989 p. S58.

Girardin et al, "Tumor Necrosis Factor and Interlukin–1 In The Serum of Children With Severe Infectious Purpura", *New England Journal of Medicine* 319(7):397–400 (1988).

Hilton et al., "A Report on the International Conference on Inflammation Held in Rome, Oct. 6–11, 1991", *DN & P*5(1):59–62 (1992).

Rosenstreich et al., "A Human Urine–Derived Interleukin1 Inhibitor: Homology with Deoxyribonuclease 1", *J. Exp. Med.* 168:1767–1779 (1988).

Takahashi K., "Basal and Clinical Investifation of Urine IL–1 Inhibitor", *Medical Journal of Hiroshima University*35(4):813–842 (1987).

Ziegler et al., "Treatment of Gram–Negative Bacteremia and Shock With Human Antiserum to a Mutant *Escherichia coli*", *New England Journal of Medicine* 307:1225–1230 (1982).

FIG. 1

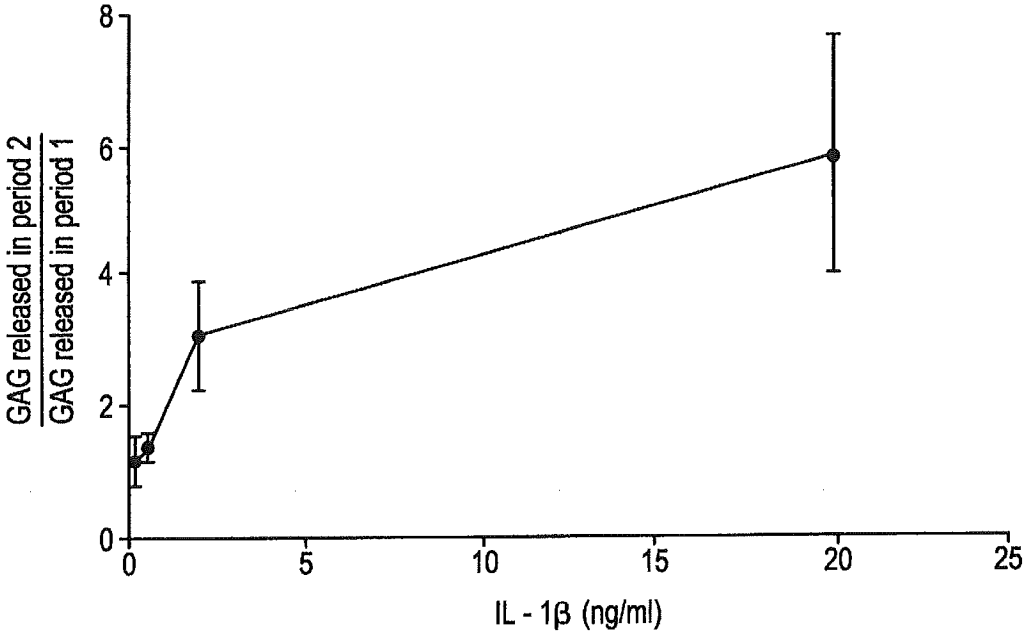

GAG released in period 2
GAG released in period 1
8
6
4
2
0
0
5
10
15
20
25
IL - 1β (ng/ml)

FIG. 2

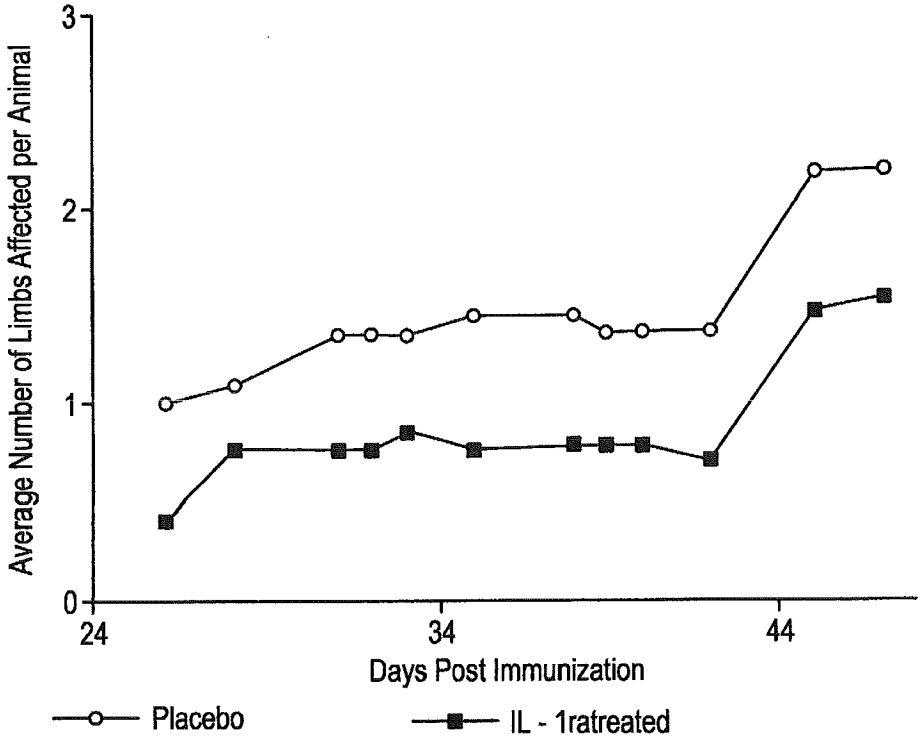

Average Number of Limbs Affected per Animal
3
2
1
0
24
34
44
Days Post Immunization
Placebo
IL - 1ratreated

FIG. 3

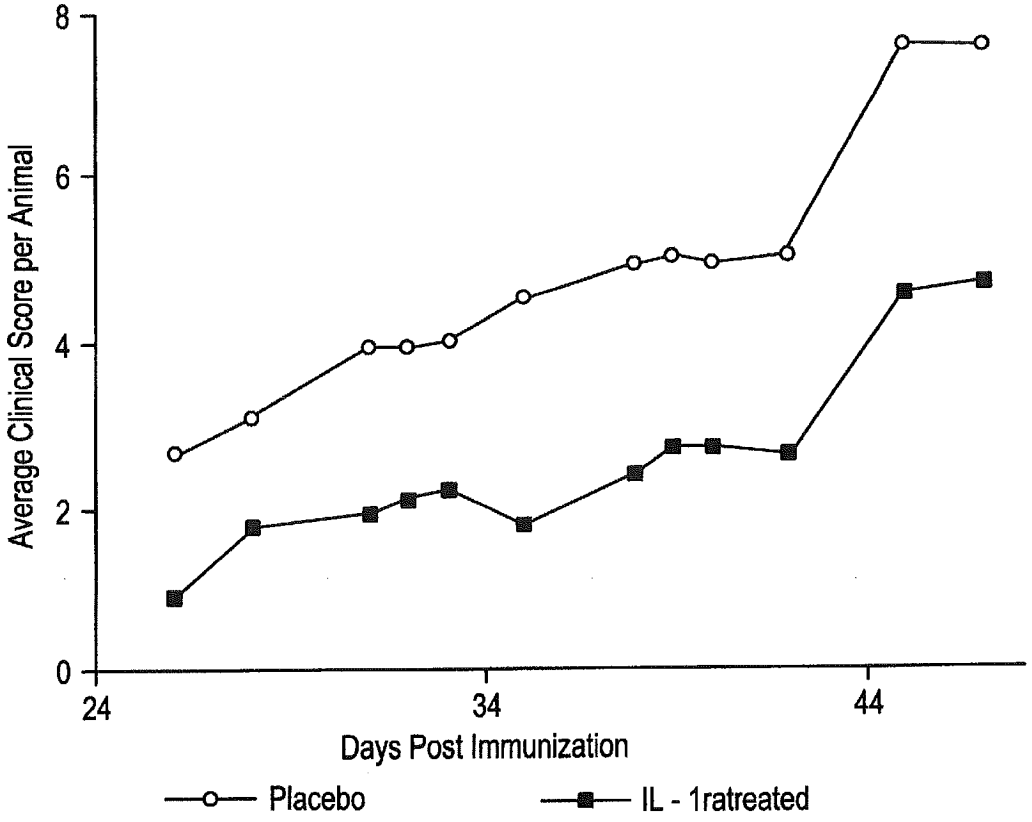

Average Clinical Score per Animal
8
6
4
2
0
24
34
44
Days Post Immunization
Placebo
IL - 1ratreated

FIG. 4

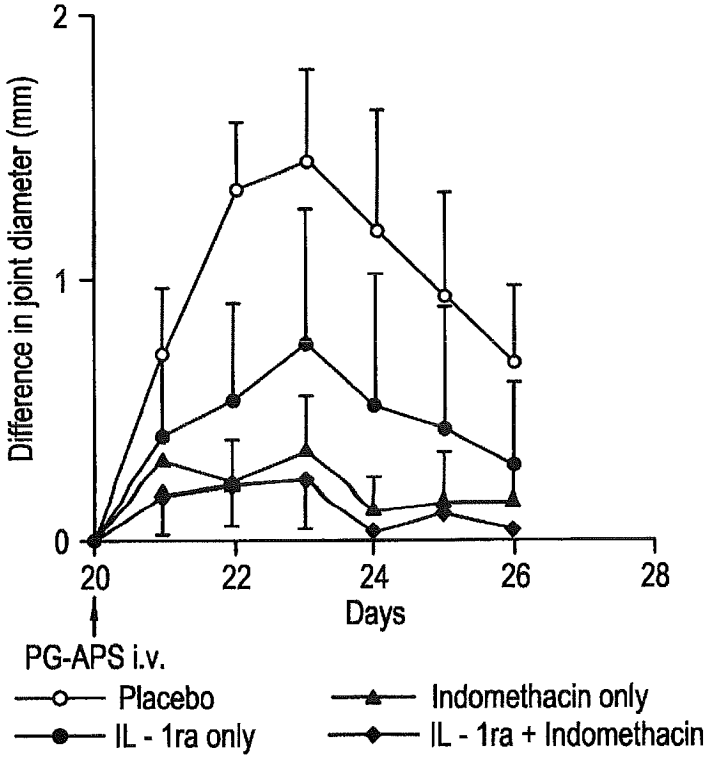

Difference in joint diameter (mm)
2
1
0
20
22
24
26
28
Days
PG-APS i.v.
Placebo
IL - 1ra only
Indomethacin only
IL - 1ra + Indomethacin

FIG. 5

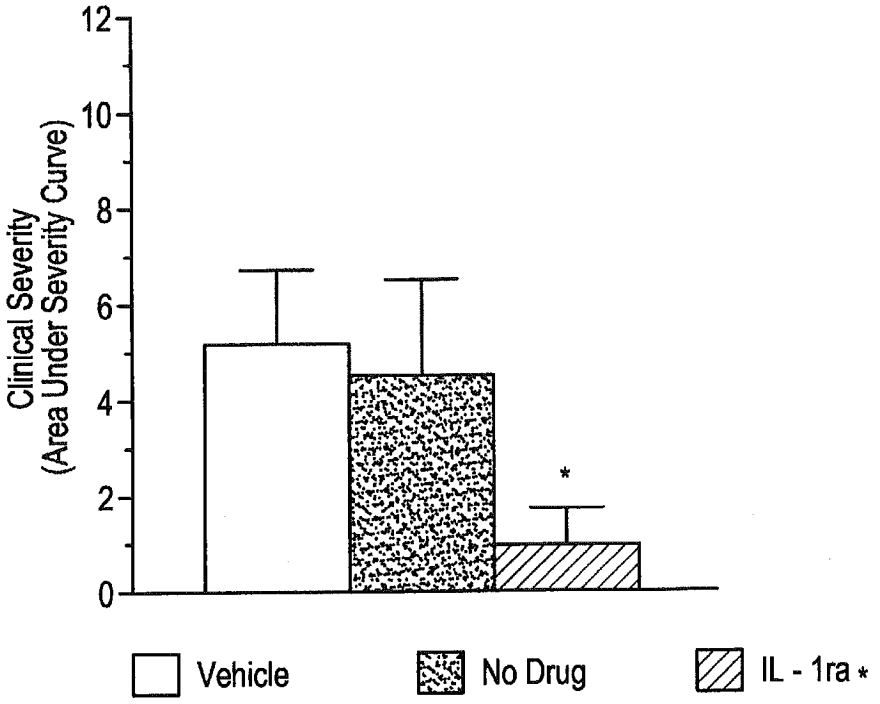

12
10
8
6
4
2
0
Clinical Severity
(Area Under Severity Curve)
*
Vehicle
No Drug
IL - 1ra *

FIG. 6

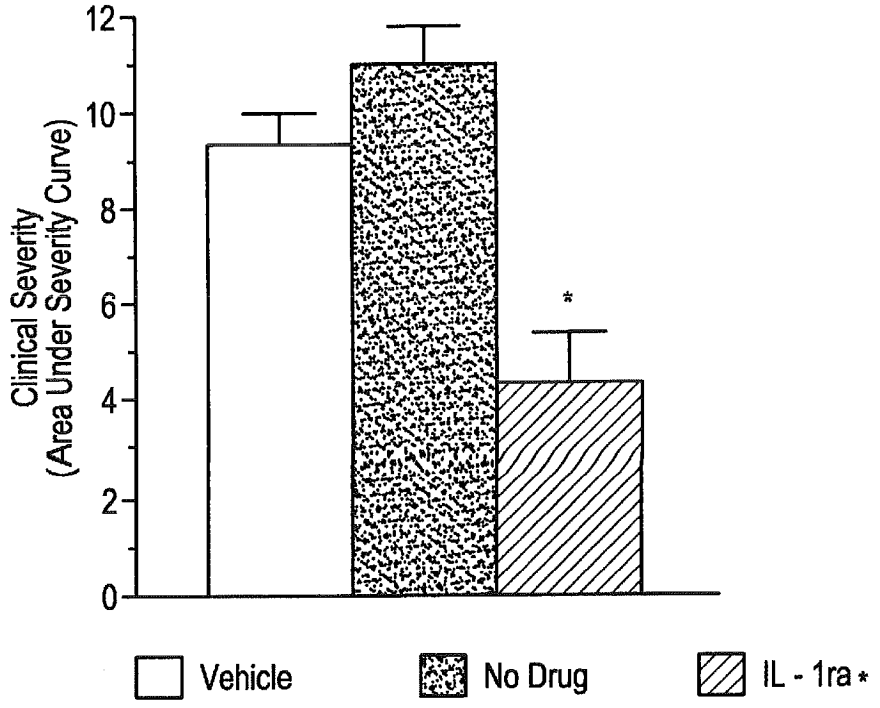
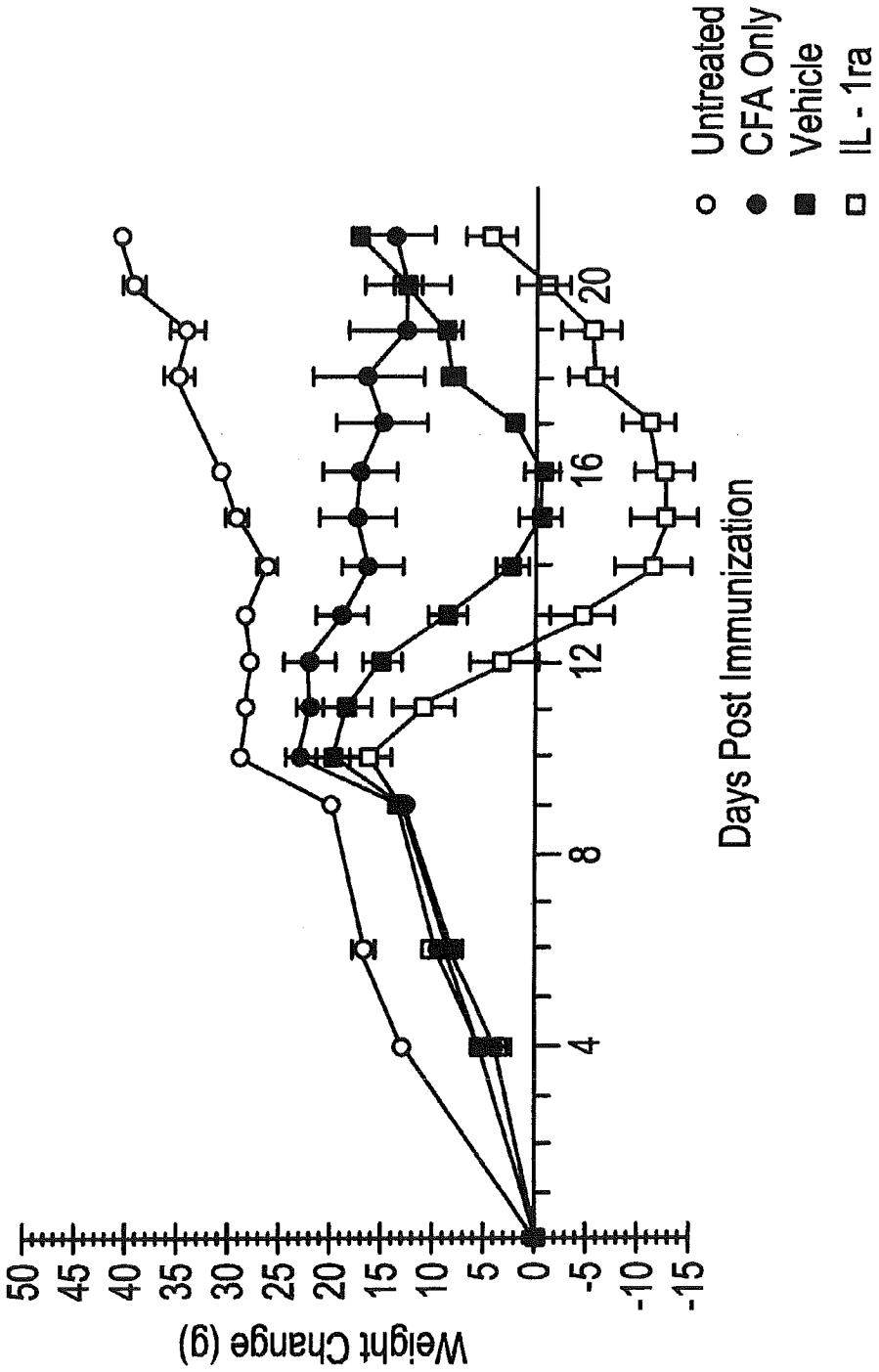

12
10
8
6
4
2
0
Clinical Severity
(Area Under Severity Curve)
*
Vehicle
No Drug
IL - 1ra *

FIG. 7


Untreated
CFA Only
Vehicle
IL - 1ra
Days Post Immunization
0
4
8
12
16
20
Weight Change (g)
50
45
40
35
30
25
20
15
10
5
0
-5
-10
-15

METHOD FOR TREATING INTERLEUKIN-1 MEDIATED DISEASES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of abandoned U.S. patent application Ser. No. 08/002,074, filed Jan. 8, 1993, abandoned U.S. patent application Ser. No. 08/171,873, filed Dec. 22, 1993, abandoned U.S. patent application Ser. No. 08/171,876, filed Dec. 22, 1993, and abandoned U.S. patent application Ser. No. 08/171,867, filed Dec. 22, 1993, now abandoned all of which are incorporated herein by reference in their entirety.

U.S. patent application Ser. No. 08/002,074 is a continuation of abandoned U.S. patent application Ser. No. 07/849,635, filed Mar. 5, 1992, which is a continuation of abandoned U.S. patent application Ser. No. 07/502,745, filed Apr. 2, 1990, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/463,888, filed Jan. 11, 1990, which is a continuation of abandoned U.S. patent application Ser. No. 07/248,521, filed Sep. 23, 1988, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/238,713, file Aug. 31, 1988, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/199,915, filed May 27, 1988.

U.S. patent application Ser. No. 08/171,873 is a continuation of abandoned U.S. patent application Ser. No. 08/047,765, filed Apr. 15, 1993, which is a continuation of abandoned U.S. patent application Ser. No. 07/895,145, filed Jun. 5, 1992, which is a continuation of abandoned U.S. patent application Ser. No. 07/524,210, filed May 16, 1990, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/502,745, filed Apr. 2, 1990, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/463,888, filed Jan. 11, 1990, which is a continuation of abandoned U.S. patent application Ser. No. 07/248,521, filed Sep. 23, 1988, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/238,713, filed Aug. 31, 1988, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/199,915, filed May 27, 1988.

U.S. patent application Ser. No. 08/171,876 is a continuation of abandoned U.S. patent application Ser. No. 08/047,762, filed Apr. 15, 1993, which is a continuation of abandoned U.S. patent application Ser. No. 07/895,153, filed Jun. 5, 1992, which is a continuation of abandoned U.S. patent application Ser. No. 07/530,553, filed May 29, 1990, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/524,210, filed May 16, 1990, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/502,745, filed Apr. 2, 1990, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/463,888, filed Jan. 11, 1990, which is a continuation of abandoned U.S. patent application Ser. No. 07/248,521, filed Sep. 23, 1988, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/238,713, filed Aug. 31, 1988, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/199,915, filed May 27, 1988.

U.S. patent application Ser. No. 08/171,867 is a continuation of abandoned U.S. patent application Ser. No. 08/097,308, filed Jul. 26, 1993, which is a continuation of abandoned U.S. patent application Ser. No. 07/936,874, filed Aug. 27, 1992, which is a continuation of abandoned U.S. patent application Ser. No. 07/678,732, filed Apr. 1, 1991, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/530,553, filed May 29, 1990, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/524,210, filed May 16, 1990, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/502,745, filed Apr. 2, 1990, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/463,888, filed Jan. 11, 1990, which is a continuation of abandoned U.S. patent application Ser. No. 07/248,521, filed Sep. 23, 1988, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/238,713, filed Aug. 31, 1988, which is a continuation-in-part of abandoned U.S. patent application Ser. No. 07/199,915, filed May 27, 1988.

BACKGROUND OF THE INVENTION

The present invention describes methods for preventing or treating a variety of diseases and deleterious medical conditions associated with interleukin-1 (IL-1).

Cytokines are extracellular proteins that modify the behavior of cells, particularly those cells that are in the immediate area of cytokine synthesis and release. One of the most potent inflammatory cytokines thought to be a key mediatory in many diseases and medical conditions is IL-1. Interleukin-1 is manufactured, although not exclusively, by cells of the macrophage/monocyte lineage and is produced in two known forms, IL-1 alpha (IL-1α) and IL-1 beta (IL-1β).

A disease or medical condition is considered an "interleukin-1 mediated disease" if the spontaneous or experimental disease or medical condition is associated with elevated levels of IL-1 in bodily fluids or tissue, or if cells or tissues taken from the body produce elevated levels of IL-1 in culture. In many cases, such IL-1 mediated diseases are also recognized by the following additional two conditions: (1) pathological findings associated with the disease or medical condition can be mimicked experimentally in animals by the administration of IL-1; and (2) the pathology induced in experimental animal models of the disease or medical condition can be inhibited or abolished by treatment with agents that inhibit the action of IL-1. In most IL-1 mediated diseases, at least two of these three conditions are met. Diseases or medical conditions that are IL-1 mediated include, for example, arthritis, inflammatory bowel disease, sepsis and septic shock, reperfusion injury, osteoporosis, asthma, insulin diabetes, myelogenous and other leukemias, psoriasis, cachexia/anorexia, multiple sclerosis, and ischemic injury, including cerebral infarctions such as cerebral palsy.

Arthritis is a chronic joint disease that afflicts and disables, to varying degrees, millions of people worldwide. The disease is typically characterized at the microscopic level by the inflammation of synovial tissue and by a progressive degradation of the molecular components constituting the joint cartilage and bone. Continued inflammation and erosion of the joint frequently lead to considerable pain, swelling, and loss of function. While the etiology of arthritis is poorly understood, considerable information has recently been gained regarding the molecular aspects of inflammation. This research has led to the identification of certain cytokines, which are believed to figure prominently in the mediation of inflammation. The involvement of interleukin-1 in arthritis has been implicated by two distinct lines of evidence. First, increased levels of interleukin-1 and of the mRNA encoding it have been found in the synovial tissue and fluid of arthritic joints. See G. Buchan et al., "Third Annual General Meeting of the British Society for

Rheumatology," London, England, Nov. 19–21, 1988 *J. Rheumatol.* 25 (Supplement 2); Fontana et al., *Rheumatology Int.,* 2:49–53 (1982); Duff et al., *Monokines and Other Non-Lymphocytic Cytokines,* M. Powanda et al., editors, pp. 387–392 (Alan R. Liss, Inc. 1988).

Second, the administration of interleukin-1 to healthy joint tissue has been shown on numerous occasions to result in the erosion of cartilage and bone. In one experiment, intraarticular injections of IL-1 into rabbits were shown to cause cartilage destruction in vivo as described by Pettipher et al., *Proc. Nat'l Acad. Sci. U.S.A.,* 83:8749–8753 (1986). In other studies, IL-1 was shown to cause the degradation of both cartilage and bone in tissue explants. Relevant references include J. Saklatavala et al., *Development of Diseases of Cartilage and Bone Matrix,* pp. 291–298 (Alan R. Liss, Inc.) and Stashenko et al., *The American Association of Immunologists,* 183:1464–1468 (1987). One generally accepted theory used to explain the causal link between IL-1 and inflammation is that IL-1 stimulates various cell types, such as fibroblasts and chondrocytes, to produce and secrete proinflammatory or degradative compounds, such as prostaglandin E2 and collagenase.

Inflammatory bowel disease ("IBD") is a term used to describe both acute and chronic inflammatory conditions of the intestinal tract tissue and encompasses two generally distinct maladies known as ulcerative colitis and Crohn's disease. Ulcerative colitis is a mucosal ulceration of the colon. Crohn's disease, which is also referred to as ileitis, ileocolitis and colitis, is a transmural inflammation that can be found throughout the general intestinal tract.

IBD is characterized by various histological features including transmural acute and chronic granulomatous inflammation with ulceration, crypt abbesses and marked fibrosis. Not all of these indications, however, will be found in all IBD cases. Spontaneous reactivation, extraintestinal inflammation and anemia are often associated with IBD. Large joint arthritis is commonly found in patients suffering from Crohn's disease.

In the molecular processes of the inflammation associated with arthritis, research has found that various cytokines appear to mediate aspects of IBD. In particular, IL-1 has been implicated as a mediating material in IBD. Again, two distinct lines of evidence lead to this conclusion. Increased levels of IL-1 have been found in affected areas of intestines from patients with IBD. Tissues from patients with active ulcerative colitis showed IL-1 levels about 15 times the level found in control samples. Tissues with active Crohn's disease showed IL-1 levels about 6 times that of control, and tissues with inactive Crohn's disease were about three times that of the control tissue samples. See, Sartor et al., *Gastroenterology,* 94:A399; see also Satsangi et al., *Clin. Exp. Immunol.,* 67:594–605 (1987); Rachmilewitz et al., *Gastroenterology,* 67:594–605 (1989)(the bioassay used to determine IL-1 concentration levels is known to also unselectively detect IL-2, IL-4, IL-6 and IL-7).

The role of IL-1 in IBD has also been implicated by studies that have shown that the perfusion of rabbit colons with IL-1 induces the production of prostaglandin and thromboxane. Comminelli et al., *Gastroenterology,* 97:1400–1405 (1989). This finding is consistent with the hypothesis described above, i.e., IL-1 is linked with the inflammation of tissues due to its stimulatory effect of producing proinflammatory or degradative compounds. Thus, it is likely that systemic and local IL-1 production initiates or contributes to the inflammatory response in IBD, and plays an active role in the pathogenesis of the disease. The systemic production of IL-1 may also be responsible, in part, for the extraintestinal inflammation associated with Crohn's disease.

Sepsis syndrome, referred to herein as "sepsis," is the systemic inflammatory response caused by microbial infection. For example, infections caused by the release of endotoxins by gram negative bacteria elicit the secretion of several cytokines including tumor necrosis factor alpha and IL-1. Sepsis, including septic shock and severe sepsis, is not caused directly by the invading microorganism. Rather, it is a result of an overwhelming cytokine response that induces pathologic changes in the host, including changes in thermoregulation, vascular permeability and resistance, cardiac function, bone marrow function, and the activity of key enzymes.

In the case of severe sepsis and septic shock, sepsis syndrome is characterized by periods of deteriorating organ function that may result in multiple organ dysfunction leading to death. Sepsis is the most common cause of death in intensive care units and statistics indicate that the incidence of the disease has substantially increased over the past decade. Septic shock for example, is characterized by various symptoms, including a drop in mean arterial blood pressure (MAP), a decrease in cardiac output, tachycardia, tachypnea, lacticacidemia and leukopenia. At present there are few treatment options for patients suffering from sepsis and septic shock, and the treatments available are generally supportive in nature rather than treatment for the pathologic condition.

That IL-1 may have a role in the mediation of sepsis and septic shock has been suggested by various studies. In one study of children suffering from gram-negative septicemia, elevated levels of IL-1 were found in 21% of the patients examined. In addition, it was shown that IL-1 serum levels were significantly higher in patients who died than in the survivors. Girardin et al., *New Engl. J. Med.,* 319:397–400 (1988); see also, Cannon et al., *Critical Care Medicine,* S58 (April 1989)(abstract).

It has also been shown that human IL-1 induces a shock-like state in rabbits. A single bolus injection of human IL-1β resulted in hypotension and several hemodynamic and hematological parameters characteristic of septic shock. For example, the mean arterial blood pressure of IL-1 injected rabbits decreased by a minimum of 19.1%. Okusawa et al., *J. Clin. Inves.,* 81:1162–1171 (1988).

Ischemic injury may occur to a tissue or organ whenever that tissue or organ is deprived of its normal blood flow. Further damage may occur when the flow of oxygenated blood is restored to that tissue. The extent and reversibility of the damage imparted depends partly on the severity of the original insult. It is possible, however, to mitigate the extent of tissue damage resulting from reperfusion by a variety of therapeutic interventions. Simpson et al., "Oxygen Radicals and Tissue Injury," *Brook Lodge Symposium - Upjohn* (B. Halliwell, ed. 1988).

Reperfusion injury is a well documented sequela to ischemic episodes in the heart, gut, kidney, liver and other organs. Simpson et al., supra; Herman et al., *FASEB J.,* 2:146–151 (1988); McDougal, *J. of Urology,* 140:1325–1330 (1988); Finn, *Kidney Int.,* 7:171–182 (1990); Schrier, *Klin. Wochenschr.,* 66:800–807 (1988); and Winchel, *Transportation,* 48:393–396 (1989). The exact pathogenesis of reperfusion injury may vary depending on the tissue affected. In the heart, for instance, reperfusion injury is accompanied by a dramatic influx of neutrophils, and these cells are thought to play a major role affecting the

reperfusion damage (Lucchesi et al., *Ann. Rev. Pharmacol. Toxicol.,* 26:201–224 (1988)). Renal ischemia and reperfusion injury, on the other hand, appear to involve an increase in tubular cell membrane permeability, increased levels of intracellular calcium, altered mitochondrial respiratory function, and the generation of free radicals. In the kidney, the role of extravasating neutrophils in affecting the reperfusion injury is less certain. McDougal, *J. Urology,* 140:1325–1330 (1988); Finn, *Kidney Int.,* 37:171–182 (1990); Schrier, *Klin. Wochenschr.,* 66:800–807 (1988); and Winchel, *Transportation,* 48:393–396 (1989).

Despite the differences in cellular participation during ischemia and reperfusion injury, there may be similarities in the underlying mechanism. IL-1 is recognized as an early stage mediator of organ injury, and may be generated by resident or newly infiltrated inflammatory cells giving rise to organ specific tissue pathology.

Current research in ischemia related brain disorders implicates enhanced synaptic release of excitatory amino acid neurotransmitters as a major contributor to brain injury. However, recent animal studies also suggest a possible role for certain cytokines as described, for example, in Relton & Rothwell, *Brain Res. Bull.,* 29:243–246 (1992). In addition, increased levels of IL-1β in cerebralspinal fluid have been detected in pathophysiological conditions including chronic relapsing experimental encephalomyelitis in guinea pig, bacterial meningitis in humans, patients with human immunodeficiency virus type-1 infection, Alzheimer's disease and in patients with head injuries. Furthermore, it has also been found that recombinant human IL-1β when injected into the striatum of rats produced extensive neuronal damage with a loss of glutamic acid decarboxylase activity as described in Rothwell & Relton, *Neurosci. Biobehav. Rev.,* 17:217–227 (1993). These studies indicate the involvement of the IL-1 receptor systems in the pathogenesis of ischemia related brain disorders, including cerebral palsy.

Cerebral palsy is a generic term defining a non-progressive static disturbance of motor function, present from birth or early life, caused by a discrete encephaloclastic insult to the central nervous system (CNS) during gestation, the perinatal period or infancy. Although most cases result from ischemic-hypoxic insults, infection, hemorrhage or trauma may occasionally result in the pathologic condition.

The lesions associated with cerebral palsy are predominantly caused by ischemic-hypoxic insults to the immature brain due to asphyxia. There are several types of cerebral palsy depending on the pattern, location and severity of the ischemic-hypoxic insult. For example, spastic diplegia results from ischemic-hypoxia necrotic lesions localized near the dorsolateral surfaces of the lateral ventricles believed to be an end-arterial zone in pre-term infants. More extensive ischemic-hypoxic insults produce significant cystic destruction of central white matter of the hemispheres (deep lesions) associated with quadriplegia and mental deficiency. Ischemic-hypoxic insults to term infants tend to affect the parasagittal and parietoccipital cortex watershed zone, hippocampus, thalamus, and cerebellar hemispheres (superficial lesions). Congenital hemiplegia results from arterial occlusion in the middle cerebral artery territory resulting in a pro-encephalic lesion of the hemisphere, or from a more diffuse and partial hemisphere insult. The extent of damage to the cerebral cortex has been correlated with an increased likelihood of seizures and abnormal intelligence. There are no known effective methods for treating cerebral palsy.

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS. MS is a progressive disease of adults characterized by relapses and remissions, often leading to progressive physical, cognitive and emotional impairment. Although the cause of MS is unknown, the pathological, genetic and immunological features of the condition have been identified and indicate that the disease has an autoimmune basis.

Studies have shown that IL-1 can augment the in vitro activation of encephalitogenic T lymphocytes and enhance adoptive transfer of experimental autoimmune encephalomyelitis (EAE). EAE is an acute or chronic relapsing inflammatory demyelinating disease of the CNS resulting from sensitization of genetically susceptible animals with neuroantigens such as myelin basic protein (MBP). EAE is an art-accepted and often used animal model for acute human MS. Evidence of the involvement of IL-1 in immune-mediated demyelination comes from in vivo EAE studies. These studies demonstrate that exogenous IL-1α can exacerbate the clinical severity and duration of the paralysis observed in the EAE animal model. Current treatment for MS include the use of steriods and more recently interferon β (IFβ). Steriods, however, are known to have many deleterious side effects when administered over a period of time.

Accordingly, a need exists for an effective, yet selective, inhibitor or IL-1 for the treatment, amelioration or prevention of arthritis, IBD, sepsis and septic shock, ischemic injury, reperfusion injury, multiple sclerosis and ischemic brain injury such as cerebral palsy and generally for use in the treatment of inflammation. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention relates to methods of treating or preventing IL-1 mediated diseases by administering to a patient in need thereof a therapeutic amount of an IL-1 inhibitor to prevent, treat or ameliorate IL-1 mediated diseases. Such IL-1 mediated diseases include, for example, arthritis, inflammatory bowel disease, sepsis and septic shock, ischemic injury, reperfusion, ischemic brain injury such as cerebral palsy and multiple sclerosis.

Particularly useful IL-1 inhibitors are proteins, and more particularly, naturally-occurring proteins since they pose a relatively low risk of producing undesirable or unforeseen side effects in patients treated with the IL-1 inhibitor. Preferably, the IL-1 inhibitors are the human proteins that naturally serve as IL-1 receptor antagonists (IL-1ra's). Also preferred are proteins that have been modified from such naturally-occurring IL-1ra's, for example by the addition of polyethylene glycol (PEG) or any other repeat polymer to increase their circulating half-life and/or to decrease their immunogenicity. In addition, the proteins can be modified by addition, deletions or substitutions in the amino acid sequence of such IL-1ra's that does not substantially reduce the biological activity of the unmodified protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the release of glycosaminoglycans (GAG) from bovine nasal cartilage in response to increasing amounts of IL-1β.

FIGS. **2** and **3** depict the inhibitory effect of IL-1ra on the pathogenesis of type II collagen-induced arthritis in mice.

FIG. **4** depicts the effects of Il-1ra on PG-APS reactivation of joint inflammation in conjunction with indomethacin.

FIG. **5** shows the in vivo effects of IL-1ra (100 mg/kg) on an animal model with EAE induced with 3 μg of MBP ($p<0.032$).

FIG. 6 shows the in vivo effects of IL-1ra (100 mg/kg) on an animal model with EAE induced with 30 μg of MBP ($p<0.01$).

FIG. 7 shows the weight change from day 0 in rats immunized with 30 μg MBP and treated with 100 mg/kg IL-1ra.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for treating or preventing various IL-1 mediated conditions. Such conditions include, for example, arthritis, inflammatory bowel disease, sepsis including septic shock, ischemic injury, reperfusion injury, ischemic brain injury such as cerebral palsy and multiple sclerosis. Other IL-1 mediated diseases include osteoporosis, asthma, insulin diabetes, myelogenous and other leukemias, psoriasis, and cachexia/anorexia. These methods are accomplished by administering a therapeutically effective amount of an IL-1 inhibitor to a patient in need thereof.

In one embodiment, the IL-1 inhibitors of the present invention are naturally-occurring proteins that serve as IL-1 receptor antagonists. These naturally-occurring proteins are particularly useful in part because they pose a comparatively low risk of producing unforeseen and undesirable physiological side effects in treated patients.

For purposes herein, a protein is deemed to be "naturally-occurring" if it or a substantially equivalent protein can be found to exist normally in healthy humans or other animals. "Naturally-occurring" proteins can be obtained by recombinant DNA methods as well as by the isolation of the proteins from cells that ordinarily produce them. Thus, "naturally occurring" also encompasses proteins that contain an N-terminal methionyl group as a consequence of expression in procaryotic cells, such as *E. coli.*

"Substantially equivalent" as used herein is defined to mean possessing a very high degree of amino acid residue homology (see generally, M. Dayhoff, *Atlas of Protein Sequence and Structure,* 5:124 (1974), specifically incorporated herein by reference), as well as possessing comparable biological activity to the naturally-occurring proteins. By "biologically equivalent", as used throughout the specification and claims, we mean compositions of the present invention that are capable of preventing IL-1 action in similar fashion, but not necessarily to the same degree, as the native IL-1 inhibitor isolated from monocytes. By "substantially homologous", as used throughout the ensuing specification and claims, is meant a degree of homology to the native IL-1 inhibitor isolated from monocyte-conditioned medium in excess of that displayed by any previously reported IL-1 inhibitors. Preferably, the degree of homology is in excess of 70 percent, more preferably in excess of 80 percent and even more preferably in excess of 90 percent. A particularly preferred group of inhibitors is in excess of 95 percent homologous with the native inhibitor. The percentage homology as described is calculated as the percentage of amino acid residues found in the smaller of the two sequences that align with identical amino acid residues in the sequence being compared when four gaps in a length of 100 amino acids may be introduced to assist in that alignment as set forth by Dayhoff, M. D. in Atlas of Protein Sequence and Structure, Vol. 5, p.124 (1972), National Biochemical Research Foundation, Washington, D.C., specifically incorporated herein by reference.

Particularly preferred IL-1ra's of the present invention are the naturally-occurring proteins that exist in vivo as regulators of IL-1 that have previously been described in U.S. Pat. No. 5,075,222 entitled "Interleukin-1 Inhibitors" (referred to herein as the '222 patent) which is incorporated herein by reference in its entirety. The proteins include glycosylated as well as non-glycosylated IL-1 receptor antagonists.

Three useful forms of Il-1ra are disclosed and described in the '222 patent. The first of these, IL-1raα, is characterized as a 22–23 kD molecule on SDS-PAGE with an approximate isoelectric point of 4.8, eluting from a Mono Q FPLC column at around 52 mM NaCl in Tris buffer, pH 7.6. The second, IL-1raβ, is characterized as a 22–23 kD protein, eluting from a Mono Q column at 48 mM NaCl. Both Il-1raα and IL-1raβ are glycosylated. The third, IL-1rax, is characterized as a 20 kD protein, eluting from a Mono Q column at 48 mM NaCl and is non-glycosylated. All three of these inhibitors were shown to possess similar functional and immunological activities. As disclosed in the '222 patent, the amino acid sequence of IL-1ra is as follows:

(U)(X) Pro Ser Gly Arg Lys Ser Ser Lys Met Gln Ala Phe Arg Ile Trp Asp Val Asn Gln Lys Thr Phe Tyr Leu Arg Asn Asn Gln Leu Val Ala Gly Tyr Leu Gln Gly Pro Asn Val Asn Leu Glu Glu Lys Ile Asp Val Val Pro Ile Glu Pro His Ala Leu Phe Leu Gly Ile His Gly Gly Lys Met Cys Leu Ser Cys Val Lys Ser Gly Asp Glu Thr Arg Leu Gln Leu Glu Ala Val Asn Ile Thr Asp Leu Ser Glu Asn Arg Lys Gln Asp Lys Arg Phe Ala Phe Ile Arg Ser Asp Ser Gly Pro Thr Thr Ser Phe Glu Ser Ala Ala Cys Pro Gly Trp Phe Leu Cys Thr Ala Met Glu Ala Asp Gln Pro Val Ser Leu Thr Asn Met Pro Asp Glu Gly Val Met Val Thr Lys Phe Tyr Phe Gln Glu Asp Glu

wherein (U) is nothing, Met or comprises an N-terminal secretion leader sequence which directs the polypeptide out of a cell in a processed form and (X) is Arg or Pro.

Methods for producing the IL-1 inhibitors are also disclosed in the '222 patent. One disclosed method consists of isolating the inhibitors from human monocytes, where they are naturally produced. A second disclosed method involves isolating the gene responsible for coding the inhibitors, cloning the gene in suitable vectors and cells types, expressing the gene to produce the inhibitors and harvesting the inhibitors. The latter method, which is exemplary of recombinant DNA methods in general, is a preferred method of the present invention. Recombinant DNA methods are preferred in part because they are capable of achieving comparatively greater amounts of protein at greater purity.

The present invention also includes modified IL-1ra's. In one embodiment, the Il-1ra is modified by attachment of one or more polyethylene glycol (PEG) or other repeating polymeric moieties as described in PCT Publication No. WO 92/16221, specifically incorporated herein by reference. In another embodiment, the IL-1ra contains an N-terminal methionyl group as a consequence of expression in *E. coli.*

Additional IL-1 inhibitors include compounds capable of specifically preventing activation of cellular receptors to IL-1. Such compounds include IL-1 binding proteins such as soluble receptors and antibodies, including monoclonal antibodies. Such compounds also include receptor antagonists and monoclonal antibodies to the receptors.

A second class of IL-1 inhibitors include the compounds and proteins that block in vivo synthesis and/or agents that affect transcription of IL-1 genes or processing IL-1 preproteins. Under certain conditions, the IL-1 inhibitor will block IL-1 induced IL-1 production.

Preferably, the above described IL-1ra's are produced by the aforementioned method in "substantially pure" form. By "substantially pure" it is meant that the inhibitor, in an

unmodified form, has a comparatively high specific activity, preferably in the range of approximately 150,000–500,000 receptor units/mg as defined in Hannum et al., *Nature,* 343:336–340 (1990); and Eisenberg et al., *Nature,* 343:341–346 (1990), both of which are specifically incorporated herein by reference. It is to be recognized, however, that derivatives of IL-1ra can have different specific activities. In a preferred embodiment of the present invention, a therapeutic composition comprising at least one IL-1ra is administered in an effective amount to patients suffering from an interleukin-1 mediated disease.

Because it is possible that the inhibitory function of the IL-1 inhibitors is imparted by one or more discrete and separable portions, it is also envisioned that the method of the present invention could be practiced by administering a therapeutic composition whose active ingredient consists of that portion (or those portions) of an inhibitor which controls (or control) IL-1 inhibition.

The therapeutic composition of the present invention is preferably administered parenterally by injection, although other effective administration forms, such as intraarticular injection, inhalant mists, orally active formulations, or suppositories, are also envisioned. One preferred carrier is physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers may also be used. In one preferred embodiment, it is envisioned that the carrier and the IL-1ra constitute a physiologically-compatible, slow-release formulation. The primary solvent in such a carrier may be either aqueous or non-aqueous in nature. In addition, the carrier can contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, or odor of the formulation. Similarly, the carrier can contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release or absorption of the IL-1ra. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dose or multi-dose form.

Once the therapeutic composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations can be stored either in a ready to use form or requiring reconstitution immediately prior to administration. The preferred storage of such formulations is at temperatures at least as low as 4° C. and preferably at −70° C. It is also preferred that such formulations containing IL-1ra are stored and administered at or near physiological pH. It is presently believed that storage and administration in a formulation at a high pH (i.e., greater than 8) or at a low pH (i.e., less than 5) is undesirable.

Preferably, the manner of administering the formulations containing IL-1ra is via an intraarticular, subcutaneous or intramuscular route. Preferably, the manner of administering the formulations containing IL-1ra is via intra-articular, subcutaneous, intramuscular or intravenous injection, suppositories, enema, inhaled aerosol, or oral or topical routes. To achieve and maintain the desired dose of IL-1ra, repeated subcutaneous or intramuscular injections can be administered. Both of these methods are intended to create a preselected concentration range of IL-1ra in the patient's blood stream. Preferably, circulating concentrations of Il-1ra range from 0.01 ng/ml to about 100 μg/ml.

A preferred dosage range for the treatment of interleukin-1 mediated arthritis is between 1 and 100 ng/ml. Accordingly, dosages are initially administered to bring the circulating levels of IL-1ra above 10 ng/ml of plasma and that, thereafter, doses are administered at a suitable frequency to keep the circulating level of IL-1ra at or above approximately 10 ng/ml of plasma. The frequency of dosing depends on the pharmacokinetic parameters of the IL-1ra in the formulation used.

A preferred dosage range for the treatment of IL-1 mediated IBD is between about 0.5–50 mg/kg of patient weight administered between about 1 and 10 times per day. More preferably, the dosage is between about 1–10 mg/kg of patient weight administered between about 3 and 5 times per day. The frequency of dosing depends on the pharmacokinetic parameters of the IL-1ra in the formulation used.

A preferred dosage range for the treatment of IL-1 mediated sepsis and septic shock is between about 1.0–200 mg/kg per day of patient body weight per 24 hours administered in equal doses between about 4–15 times per 24 hours. In a more preferred embodiment, the dosage is between about 10–120 mg/kg per day of patient body weight administered in equal doses every 24 hours. In the most preferred embodiment, 100 mg/kg of patient body weight per 24 hours is equally administered every 2 hours. The frequency of dosing depends on the pharmacokinetic parameters of the IL-1ra in the formulation used.

In an additional preferred mode for the treatment of IL-1 mediated sepsis and septic shock, an initial bolus injection of IL-1ra is administered followed by a continuous infusion of IL-1ra until circulating IL-1 levels are no longer elevated. The goal of the treatment is to maintain serum IL-1ra levels between 2–20 μg/ml for this period. In a preferred embodiment of this mode, an initial bolus of IL-1ra is administered followed by the continuous administration of IL-1ra of between about 5–20 μg/kg of patient body weight per minute until circulating IL-1 levels are not longer elevated. Serum IL-1β levels may be ascertained by commercially available immunoassay test kits. The initiation of treatment for IL-1 mediated sepsis and septic shock should be begun, under either mode of treatment, as soon as possible after septicemia or the chance of septicemia is diagnosed. For example, treatment may be begun immediately following surgery or an accident or any other event that may carry the risk of initiating sepsis or septic shock.

A preferred dosage range for the treatment of IL-1 mediated ischemia and reperfusion injury is between about 1–50 mg/kg of patient weight administered hourly. In a preferred embodiment, an initial bolus of about 15–50 mg/kg of IL-1ra is administered, followed by hourly injections of about 5–20 mg/kg. The frequency of dosing depends on the pharmacokinetic parameters of the IL-1ra in the formulation used.

A preferred dosage range for the treatment of IL-1 mediated multiple sclerosis is between about 10–100 mg/kg administered to maintain a blood level of circulating IL-1ra levels between 2–20 μg/ml for the length of the relapse. The frequency of dosing depends on the pharmacokinetic parameters of the IL-1ra in the formulation used.

A preferred dosage range for the treatment of IL-1 mediated cerebral infarctions such as cerebral palsy is between about 10–100 mg/kg by infusion to sustain a high level of IL-1ra levels at 2–20 μg/ml. The frequency of dosing depends on the pharmacokinetic parameters of the IL-1ra in the formulation used.

It is also contemplated that certain formulations containing IL-1ra are to be administered orally. Preferably, IL-1ra is encapsulated for oral administration. The encapsulated IL-1ra can be formulated with or without those carriers and excipients customarily used in the compounding of solid dosage forms. Preferably, the capsule is designed so that the active portion of the formulation is released at that point in

the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional excipients can be included to facilitate absorption of the IL-1ra as well as diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents and binders.

When used for the treatment of IL-1 mediated IBD, the administration of IL-1ra can also be accomplished in a suitably formulated enema.

Regardless of the manner of administration, the specific dose is calculated according to the approximate body weight or surface area of the patient. Other factors in determining the is appropriate dosage can include the disease or condition to be treated or prevented, route of administration and the age, sex and medical condition of the patient. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of skilled in the art and is without undue experimentation, especially in light of the dosage information and assays disclosed herein. These dosages can be determined through the use of known assays for determining dosages used in conjunction with appropriate dose-response data.

The pharmaceutical compositions of the present invention can be used for veterinary as well as human applications. Accordingly, the term "patient" is intended to encompass animals as well as humans.

The following examples are intended to illustrate, but not limit, the present invention.

EXAMPLE 1

Effects of Human IL-1 Inhibitor on Cultured Bovine Nasal Cartilage Explant

Numerous in vitro and in vivo methods have been used to study the progression of arthritis. One in vitro model which has proven to be especially useful in this regard is cultured cartilaginous tissue explant. In fact, this model has been used in the past to demonstrate that IL-1 is a powerful mediator of cartilage destruction and, therefore, a propitious target for intervention in arthritic joint erosion. (See generally, G. Buchan et al., Third Annual General Meeting of the British Society for Rheumatology, London, England, Nov. 19–21, 1988, *PR. J. Rheumatology,* Supplement 2 (1986); Fontana et al., *Rheumatol. Int.* 2:49–53 (1982); J. Saklatvala et al., *Development of Diseases of Cartilage and Bone Matrix,* pp. 291–298 (Alan R. Liss); P. Stashenko et al., *The American Association of Immunologists,* 138:1464–1468 (1987); Dodge et al., *J. Clin. Invest.,* 83:647–661; J. Sandy et al., *J. Orthopedic Res.,* 4:263–272; Saklatavala et al., *The Control of Tissue Damage* pp. 97–108 (Elsevier Science Publ.); Campbell et al., *Biochem. J.,* 237:117–122; Tyler, *Biochem. J.,* 225:493–507; and Eastgate et al., Sixth International Lymphokine Workshop, 7(3) :338).

The cartilage explant model essentially as described by Steinberg et al., *Biochem. J.,* 180:403–412, incorporated herein by reference, was used in the experiments reported in this Example to demonstrate the mitigating effect of IL-1ra on IL-1 mediated cartilage breakdown. While bovine nasal septum was used here as the source of cartilaginous tissue, articular cartilage of the type described in Typer et al., *Br. J. Theumatol.,* 24(1):150–155, incorporated herein by reference, can also be used.

A. Preparation of Cartilage

Bovine nasal septum was removed from freshly sacrificed yearling steers and placed on ice. The tissue was then scrubbed with Povidone-Iodine prep solution (1-ethanol-2-pyrolidinone homopolymer with iodine, obtained from Medline Industries (Mundelein, Ill.). The mucosa and perichondrium were then removed. The remaining cartilaginous septum was then immersed in a 5% (v/v) solution of Povidone/Iodine for one hour at room temperature.

The following procedures were then performed aseptically in a laminar flow hood. The septa were repeatedly rinsed with Grey's Balanced Salt Solution (GIBCO laboratories, Grand Island, N.Y.). The cartilage sheet was then placed on a sterile surface, and uniform 8 mm plugs were removed using a standard cork borer. Approximately 1–2 mm of the top and bottom surfaces were removed using a razor blade. The plugs were then held in the Grey's Balanced Salt Solution. Plugs taken from different steers were kept separately.

Each plug was then sectioned into several 0.8 mm disks. The cutting device used was an aluminum block of the type described by Steinberg et al., *Biochem. J.,* 180:403–412. The disks produced were consistently between 40 and 50 mg wet weight. The disks were kept in culture in Delbecco's Modified Eagle Medium (DMEM) plus 10% fetal calf serum, plus penicillin, streptomycin and neomycin, hereinafter referred to as the "medium." The cultures were maintained in a 37° C. incubator with 5% CO_2.

Representative disks from each steer were then tested for their ability to respond to IL-1β as indicated by the release of glycosaminoglycans (GAG) into the culture medium. Glycosaminoglycans are released from a cell once the cell matrix has been degraded. The presence of GAG was detected using 1, 9-dimethylenethylene blue as described by Ferndale et al, *Connective Tissue Research,* 9:247–248, incorporated herein by reference. Disks that responded to 5 ng/ml of IL-1β by increasing output of GAG two fold or greater as compared to an unstimulated basal rate were selected for use in the following experiments. These disks are hereinafter referred to as "IL-1β responsive disks."

B. IL-1 Dose Response

This preliminary experiment was performed to determine whether a dose response curve exists to increasing amounts of IL-1β. First, several of the IL-1β responsive disks were sectioned into quarter slices. The remainder was set aside for later experiments. Because responses to IL-1 frequently vary from animal to animal, disk to disk, and slice to slice, the steps of this experiment were designed so that each slice served as its own control.

Second, each slice was incubated in one well of a 48 well tissue culture cluster (Costar, Cambridge, Mass.) with a constant volume of the previously described medium. After 48 hours, the amount of GAG present in the supernatant of each culture was measured. This amount was then normalized for each culture in terms of μg GAG per mg wet weight of tissue. In this manner, a basal rate of GAG release in the absence of IL-1 was established for each slice.

Third, the supernatants from all the cultures were discarded and replaced with fresh medium containing differing amounts of IL-1β. The IL-1β was produced in-house (J. Childs, notebook 935, pages 49–52) and after characterization, was utilized in all experiments calling for its use. After a 48 hour incubation with IL-1β, the supernatants from the cultures were recovered, and the amount of GAG present in each was measured. These amounts were normalized for each culture as above. The basal rates were then subtracted from the IL-1β induced rates. The results are reported in FIG. 1. As FIG. 1 clearly indicates, the release of GAG from the cartilaginous tissue is dependent on the amount of IL-1β administered.

Because 5 ng/ml of IL-1β caused an easily measurable increase in GAG release during the 48 hour period of culturing, this concentration was used in the following experiment.

C. Effects of rIL-1ra on IL-1 Induced GAG Release

Several of the remaining IL-1β responsive disks were next sectioned into quarter slices. As above, each slice was used as its own control.

Each slice was then incubated with a constant volume of the previously-described medium for 48 hours in a 48 well tissue culture cluster. A basal rate of GAG release was determined for each slice. Next, the supernatants from the cultures were discarded and replaced with fresh medium containing 5 ng/ml of IL-1β and differing amounts of recombinantly produced IL-1ra (rIL-1ra). After a 48 hour incubation, the supernatants were recovered, and the amounts of GAG were measured. These amounts were normalized for each culture by dividing the rIL-1ra/IL-1β stimulated GAG release rate by the basal GAG release rate. The results are summarized in Table 1. In all cases the concentration of IL-1β is 5 ng/ml (n=6).

TABLE 1

THE EFFECT OF RECOMBINANT IL-1ra ON IL-1β INDUCED DEGRADATION OF BOVINE NASAL CARTILAGE

[IL-1ra]	[IL-1ra] / [IL-1β]	Fold Stimulation period II/periodI (+/–standard deviation)
0		4.02 ± 1.7
5 ng/ml	1	2.4 ± 0.47
10 ng/ml	2	1.7 ± 0.4
25 ng/ml	5	1.3 ± 0.4
50 ng/ml	10	1.0 ± 0.2
150 ng/ml	30	1.1 ± 0.2

The results show that the release of GAG from cartilaginous tissue was sharply curtailed by an increase in the concentration of rIL-1ra relative to that of IL-1β. For instance, a ten times molar excess of rIL-1ra over IL-1β (the molecular weights of IL-1β and rIL-1ra are both approximately 17 kD) was sufficient to return the GAG release rate to the basal level. Similarly, a 1.5 times molar excess of rIL-1ra over IL-1β was sufficient to reduce the stimulation of GAG release to 50% of that observed in the presence of IL-1β alone. These results were reproduced using cartilage derived from several different steer.

D. Lack of Cytotoxicity of rIL-1ra

To show that rIL-1ra is noncytotoxic, slices from the remaining IL-1β responsive disks were exposed to varying amounts of rIL-1ra in the absence of IL-1β. The rate of GAG release was the same as when neither rIL-1ra nor IL-1β was present.

Next, to show that the effects of IL-1ra are reversible, rIL-1ra was removed from the supernatants of culturing slices and IL-1β was added. The slices responded as they did in the IL-1β dose response experiment. Similar results occurred when cartilage that had been treated with IL-1β and a sufficient concentration of rIL-1ra to completely block the action of IL-1β was subsequently exposed to IL-1β alone.

EXAMPLE 2

Effects of IL-1ra on Collagen-Induced Arthritis in Mice

Type II collagen-induced arthritis in mice bears many resemblances to human rheumatoid arthritis and has been used for several years to study certain aspects of that disease (Stuart et al., *FASEB J.*, 2(14):2950–2956 (1988)). The potential involvement of IL-1 in rheumatoid arthritis has been noted by Stimpson et al., *J. Immunol.*, 140:2964–2969 (1988).

The purpose of this experiment was to demonstrate that systemic administration of rIL-1ra has a mitigating effect on the pathogenesis of type II collagen-induced arthritis in mice.

Twenty-four mice DBA/1 mice, purchased from Jackson Laboratories, were immunized with 0.1 mg chick type II collagen in Freund's complete adjuvant. At day 14 post immunization, the animals were randomly subdivided into two groups of twelve animals each. The experimental group was injected intraperitoneally twice daily with approximately 0.1 mg rIL-1ra/kg/injection. The injections continued until the animals were sacrificed at day 47 post immunization (i.e., after 34 days of dosing). Control animals were injected with an equal volume of vehicle (10 mM sodium phosphate, 150 mM sodium chloride) on the same schedule.

Affected limbs were counted and clinical scoring was performed approximately three times weekly during the in-life portion of the experiment. Clinical scores from each animal represent, on a 0–4 point basis, the severity of arthritis sustained by each paw as assessed by blinded observers. The clinical scores for each animal from day 26, when the first signs of clinically observable arthritis were noted, through day 47, when the animals were sacrificed. These results are graphed as a function of time in FIGS. **2** and **3**, respectively. As can clearly be seen, the incidence and severity of the disease were slowed down considerably by the administration of rIL-1ra.

EXAMPLE 3

Effects of Human IL-1 Inhibitor on Streptococcal Cell Wall-Induced Reactivation of SCW-Induced Arthritis in Rats

Regarding streptococcal cell wall-induced arthritis, R. L. Wilder in *Immunopathogenetic Mechanisms of Arthritis,* Chapter 9 entitled "Experimental Animal Models of Chronic Arthritis" comments "the clinical, histological and radiological features of the experimental joint diseases closely resemble those observed in adult and juvenile rheumatoid arthritis."

The experiments described below employs the model disclosed in Esser et al., *Arthritis and Rheumatism,* 28:1401–1411 (1985), specifically incorporated herein by reference. Briefly, streptococcal cell wall (SCW) is injected intraarticularly into the ankle joint of Lewis rats. Saline is injected into the contralateral joint to provide a control. After a period of twenty days, in which the initial inflammation dies away, SCW is again administered, this time by intravenous injection. This dose of SCW is insufficient to cause joint inflammation by itself and, therefore, has little or no effect on the saline injected ankle. However, this dose is capable of reactivating inflammation and joint destruction in the ankle previously injected with SCW. To assess the extent of inflammation following the second administration of SCW, the dimensions of the ankle joint are measured daily.

In one of many experiments performed with the above described model, two groups of twelve rats were used. Each animal was injected in the right ankle with SCW (1.8 μg rhamnose equivalence) and in the left ankle with an equal volume of pyrogen-free saline. Ankle dimensions were measured on days 1 through 6.

On day 20, one group of rats was injected intraperitoneally with 1 mg/kg IL-1ra in an aqueous vehicle; the other group was injected intraperitoneally with an equal volume of the vehicle solution only. One hour later, each animal was injected intravenously with SCW (100 μg rhamnose equivalence). Ten minutes later, the treatment group was injected intraperitoneally with 1 mg/kg IL-1ra, and the control group was injected with vehicle alone. Subcutaneous injections of IL-1ra at 1 mg/kg were given at 2 and 6 hours post SCW administration and were repeated every 6 hours thereafter for the next 3 days.

Table 2 shows the dimensions of the saline injected and SCW injected ankles for both the treatment group and the control group over the course of the experiment.

TABLE 2

ANKLE JOINT DIAMETER OF RATS INJECTED WITH SCW AND TREATED WITH IL-1ra OR SALINE ACCORDING TO PROTOCOL IN EXAMPLE 3

	Joint Diameter (mm) (±SD)							
	SCW Injected Joints				Saline Injected Joints			
Day	IL-1ra	SD	Saline	SD	IL-1ra	SD	Saline	SD
0	5.96	.12	6.02	.10	5.95	.17	5.96	.16
1	7.95	.33	7.73	.36	5.94	.15	5.94	.13
2	7.44	.28	7.42	.27	5.98	.11	5.95	.17
3	7.20	.39	7.23	.27	6.00	.12	6.01	.07
6	6.78	.27	6.64	.29	6.06	.09	6.06	.13
10	6.58	.34	6.63	.18	6.00	.12	5.85	.16
14	6.44	.21	6.36	.17	5.99	.08	5.90	.17
20	6.46	.18	6.52	.14	5.91	.11	5.87	.20
21	7.34	.36	7.78	.31	5.73	.18	5.78	.12
22	8.31	.58	8.70	.43	5.85	.16	5.96	.22
23	8.55	.81	9.06	.42	6.02	.19	5.99	.16
24	8.23	.71	8.56	.39	6.03	.13	5.94	.20
25	8.00	.56	8.16	.43	6.05	.12	6.06	.17
28	7.48	.40	7.71	.30	6.04	.13	5.98	.13

As expected, the SCW treated ankles in both groups swelled in response to the intravenous injection of SCW. However, the response differed between treatment groups. The ankles in the control group swelled by about 30% of their initial dimensions over the first 3 days, whereas the ankles in the treatment group swelled only by 14% over the same period. Moreover, on days 1 through 5 post-intravenous injection of SCW, there was a statistically significant ($p<0.001$ by a two-tailed t-test for independent means) difference in the dimensions of the SCW-treated and contralateral control ankles of both groups.

On day 8, the rats were sacrificed and both ankles were fixed in formalin. The fixed joints were decalcified, stained, and examined. Significant differences in cartilage erosion, bursitis, periostitis, and synovitis were found between the control group and the treatment group. Some of these differences are set forth in Table 3.

TABLE 3

EFFECTS OF IL-1ra ON JOINT HISTOPATHOLOGY FOLLOWING SCW REACTIVATION OF JOINT INFLAMMATION (1 mg/kg 4 times daily on day 20 through 23)

	Placebo Group		IL-1ra Group		
Pathology	Positives/12	Score	Positives/12	Score	P
Cartilage Erosion	10	1.0 ± .6	3	0.25 ± .45	.0023

TABLE 3-continued

EFFECTS OF IL-1ra ON JOINT HISTOPATHOLOGY FOLLOWING SCW REACTIVATION OF JOINT INFLAMMATION (1 mg/kg 4 times daily on day 20 through 23)

	Placebo Group		IL-1ra Group		
Pathology	Positives/12	Score	Positives/12	Score	P
Bone Erosion	3	0.25 ± .45	2	0.17 ± .39	NS
Bursitis	11	0.92 ± .29	3	0.25 ± .45	.0003
Periostitis	9	0.75 ± .45	12	0.25 ± .45	.013
Synovitis	12	2.21 ± .84	12	1.08 ± .47	.00052
PMN	12	1.0	12	1.0	NS

EXAMPLE 4

Effects of Human IL-1 Inhibitor on Formalin-Immune Complex Induced IBD

The rabbit model of formalin-immune complex IBD has been used to investigate the role of arachidonic acid-derived inflammatory mediators and to evaluate therapeutic strategies in IBD. Zipser et al., supra; Brown et al., *Gastroenterology,* 92:45–59 (1987); Schumert et al., *Prostaglandins,* 36:565–577 (1988), all incorporated herein by reference.

The experiment described below employed the model disclosed in Zipser et al., supra. This model creates symptoms analogous to active ulcerative colitis, and is briefly summarized as follows: formaldehyde is administered via a catheter into the colon of rabbits and after a period of time the animals receive an injection of immune complexes in antigen excess. Time studies following the induction of IBD are conducted by sacrificing the animals after 48 hours and removing the colons. The colons are then histologically assessed. The effect of treatment with IL-1ra's prior to and after the induction of IBD on inflammation, edema and necrosis was compared with non-treated control animals.

A. Induction of IBD

Inflammation was induced in the distal colon of male New Zealand rabbits (2.2–2.5 kg) using a modification of the immune complex method of colitis described in Kirsnew et al., *Trans. Assoc. Am. Physicians,* 70:102–119 (1957); Hodgson et al., *Gut,* 19:225–32 (1978), which are incorporated herein by reference. Four ml of 0.45% (v/v) unbuffered formaldehyde (Electron Microscopy Sciences, Washington, Pa.) was administered via a catheter inserted 10 cm into the distal colon of anesthetized rabbits (xylazine and ketamine). Two hours later, animals received 0.85 ml of immune complexes in antigen excess through an ear vein. The complexes were prepared by incubating human serum albumin (500 μg/ml) with rabbit anti-human antisera (ICN Immunogiologicals, Costa Mesa, Calif.), decanting the supernatant, and redissolving the precipitated immune complexes with an albumin solution (6 mg/ml) as described in Zipser et al., supra.

Histologic evaluation was performed on a minimum of two longitudinal sections from each colon. All colon samples were examined in a blind fashion by a single pathologist. The mucosa and submucosa were separately evaluated for infiltration of acute inflammatory cells (neutrophils and eosinophils). A semiquantitative score of leukocytes (L) per high power field (HPF) was determined for each area examined using the following quantitations: 0=0 or 1; 0.5=2–9; 1=10–20; 1.5=21–30; 2=31–40; 2.5=

41–50; 3=51–65; 3.5=66–80; 4=>81 L/HPF. At a minimum, eight HPFs of mucosa and submucosa from each specimen were separately evaluated in each section. The inflammatory index was calculated by adding the averaged score for the mucosal and submucosal evaluations. Edema was semiquantitatively assessed on a scale of 0 to 4. Necrosis was expressed as the percent of mucosa involved. After the administration of formalin, followed by immune complexes, the distal colon develops acute inflammation. This inflammation is characterized by infiltration of neutrophils primarily into the mucosa and submucosa, mucus depletion, crypt abscesses, edema and scattered areas of mucosal necrosis, progressively increased from 0.3±0.1 (0 hours) to 4.5±0.7 (48 hours) ($p<0.001$), from 0.3±0.1 to 3.6±0.3 ($p<0.001$) and from 0% to 89% ($p<0.001$), respectively. A subsequent decrease in these parameters was observed 96 hours after the induction of IBD ($p<0.01$ versus 48 hours).

B. Treatment with IL-1ra's

A group of animals were treated intravenously with IL-1ra (5 mg/kg; n=8) or the vehicle alone (n=10) at six time points: 2 hours before and 1, 9, 17, 25, 33 hours after the administration of the immune complexes. The rabbits were sacrificed 48 hours after the induction of IBD and the colon tissue analyzed for inflammation.

Treatment of rabbits with IL-1ra significantly reduced inflammatory index from 3.2±0.4 to 1.4±0.3 ($p<0.02$), edema from 2.2±0.4 to 0.6±0.3 ($p<0.01$) and necrosis from 43±10% to 6.6±3.2% ($p<0.03$) compared to vehicle-treated IBD animals. This result shows that several of the indications of IBD may be significantly lessened by treatment with IL-1ra.

EXAMPLE 5

Bacterial Cell Wall Induced IBD in Rats

Unlike many other IBD models, the bacterial cell wall induced IBD model shows most of the indications for chronic IBD or Crohn's disease. In addition to the formation of chronic granulomatous response, this model is subject to spontaneous reactivation, anemia and extraintestinal inflammation.

The Bacterial Cell Wall model essentially as described by Sartor et al., supra., was used in this Example to demonstrate the mitigating affect of IL-1ra on IL-1 mediated IBD. The experiment was performed generally as follows: the IBD is induced in rats by the intravenous injection of a sterile sonicate of peptidoglycan polysaccharide from group A streptococci. Transient petechial hemorrhage of the colon appears within 2–3 minutes and resolves by 48–72 hours after injection. A sample group of animals were treated with IL-1ra following induction of IBD, and after a period of time the animals were sacrificed, the colons removed and gross pathology evaluated.

C. Induction of IBD

The bacterial cell wall material was prepared according to the procedures set forth in Stimpson et al., *Infect. Immun.* 51:240–249 (1986), incorporated herein by reference. Lewis rats are given subsercosal injections with streptococcal cell walls. The injections result in both local and systemic disorders that include bowel adhesions and nodules, an increased liver weight and hepatic nodules, a reduced hematocrit and hemoglobin level, and increased white blood cell count (WBC), a reduced growth rate, and a joint swelling characteristic of arthritis (see Sartor et al., *Gastroenterology,* 89:587–595 (1985), incorporated herein by reference). Three separate protocols for treatment with IL-1ra were performed with this model and reductions in nodules and adhesions have been observed in all of them. In the last two protocols, the reductions in adhesions were statistically significant.

D. Protocol A

Two groups of 12 rats were used. On day 1, both were injected with 15 μg total of streptococcal cell wall-derived peptidoglycan polysaccharide (SCW PG-APS), at 7 sites; 3 areas of the cecum, 2 areas of the Peyer's patches, and two areas of the ductal ileum. On day 11, overt signs of the disease appeared including joint swelling, diarrhea, and bloody nose. At this time, one group was dosed subcutaneously with IL-1 ra (8 mg/kg) every 12 hours and the second group was treated identically with placebo (PBS). On each day the size of the ankle joints were measured. On day 18, the animals were sacrificed and the intestines were scored on a scale of 0 to 4 for the presence of granulomas and adhesions (Table 4). The IL-1ra group had fewer nodules and adhesions. The IL-1ra group also had smaller livers as well as a reduced white blood cell count (WBC).

TABLE 4

EFFECTS OF IL-1ra ON SCW-INDUCED ENTEROCOLITIS IN THE RAT

	Intestinal Adhesions	Cecal Nodules	Liver Weight (gm)	WBC
IL-1ra	1.7	1.8	16.9	48.8
PBS	2.2	2.4	18.6	57.7
p value for comparison of groups	0.14	0.10	0.19	0.13

E. Protocol B

The protocol was similar to that used in Protocol A except that the amount of PG-APS used was reduced to 12.5 μg and the treatment with IL-1ra was started at day 8. As in Protocol A, reductions in cecal nodules, intestinal adhesions, liver weights and WBC were observed (Table 5). The reduction in adhesion was significant at the $p<0.02$ level.

TABLE 5

EFFECTS OF IL-1ra ON SCW-INDUCED ENTEROCOLITIS IN THE RAT

	Intestinal Adhesions	Cecal Nodules	Liver Weight (gm)	WBC
IL-1ra	1.4	1.7	13.3	35.1
PBS	2.2	2.3	14.1	35.8
p value for comparison of groups	0.017	0.077	0.23	0.43

F. Protocol C

The protocol used was again similar to that in Protocol A except that the amount of PG-APS was reduced to 12.5 μg (as in Protocol B) and the treatment group was started on IL-1ra 8 mg/kg subcutaneous (s.c.) and 2 mg/kg intravenously (i.v.) immediately following the PG-APS injection. Further IL-1ra injections (8 mg/kg) s.c. were given at 4, 10 and 18 hours on day 1, every 8 hours on day 2, and then every 12 hours for the duration of the experiment. Five animals in each group were sacrificed at day 3, and the remainder were sacrificed at day 18 for examination of gut lesions (Table 7).

TABLE 6

EFFECTS OF IL-1ra ON SCW-INDUCED ENTEROCOLITIS IN THE RAT

	Intestinal Adhesions	Cecal Nodules	Liver Weight (gm)	WBC
IL-1ra	0.8	0.9	0.047	10.7
PBS	1.8	1.0	0.049	10.3

TABLE 6-continued

EFFECTS OF IL-1ra ON SCW-INDUCED ENTEROCOLITIS IN THE RAT

	Intestinal Adhesions	Cecal Nodules	Liver Weight (gm)	WBC
p value for comparison of groups	0.07	0.30	0.24	0.31

On day 3 there was a significant reduction in a global parameter representing gut lesions and a reduction in adhesions that approached significance (p=0.07). In the group sacrificed at day 18, the results were confused because no disease appeared in one of the animals in the control group. However, the reduction in adhesions in the IL-1ra group was still significant at the p<0.02 level and there was also a significantly greater weight gain in the IL-1ra group.

EXAMPLE 6

NSAID Induced IBD in Rats

In an attempt to determine whether the anti-inflammation effects of IL-1ra would be additive with those of NSAIDs, rats were treated with indomethacin after the intravenous injection of PG-APS as described in Example 3 above (2 mg/kg at the time of reactivation at 12, 24 and 36 hours post activation, and every 12 hours up to 6 days), IL-1ra (2 mg/kg at 2 and 6 hours, then every 6 hours up to 36 hours and every 12 hours up to 7 days) or a combination of the two drugs as shown in FIG. **4**. The group on indomethacin alone showed a greater reduction in joint swelling than that on IL-1ra alone. However, the indomethacin group was sick and two animals died during the course of the experiment. The group receiving both drugs did even better than the group on indomethacin alone; the joint swelling was less, and the difference between the two groups was statistically significant on day 4 at p<0.03 and on day 7 and 8 at p<0.06. No animals were sick in this group and there were fewer ulcerations in the mid small intestines. Ulceration of the mid small intestine is a complication in patients on chronic oral NSAIDs. It appears, therefore, that IL-1ra alleviates some of the IBD-like complications of NSAIDs.

Table 7 shows the effects of IL-1ra on both the intestinal symptoms—ulcers, adhesions, intestinal thickening and myleloperoxidase (MPO) levels—and systemic symptoms—hematocrit (HCT), hemoglobin (HgB) and WBC levels—associated with the NSAID treatment of PG-APS induced arthritis.

TABLE 7

EFFECTS OF IL-1RA TREATMENT ON INDOMETHACIN-INDUCED GUT INJURY IN THE RAT

		Ulcers			Intestinal	MPO	HCT	Hgb	WBC
Treatment	Deaths	#	% Area	Adhesions	Thickening	u/g	%	(g/dl)	($10^3/\mu l$)
Saline	0/9	0	0	0/9	0/9	0.005(.001)	42	15	6
IL-1ra	0/10	0	0	0/10	0/10	0.01(.01)	41	14	8
Indomethacin	2/9	1.3(±0.6)	7.1(±2.7)	2/7	4/7	0.06(.02)	37	13.5	11
Indomethacin + IL-1ra	0.8	0.6(±0.4)	2.8(±1.9)	3/8	2/8	0.03(.02)	40	14	11

EXAMPLE 7

Effects of Human IL-1ra on Endotoxin Induced Septic Shock

Endotoxin induced septic shock studies were conducted on Blue Chinchilla rabbits. The experimental protocol did not focus on any indications for the induced septic shock other than group mortality. Rabbits were used in the study because their sensitivity to pyrogenic and metabolic effects of endotoxin and other bacterial products are similar to those of human subjects.

Shock was induced by a single intravenous injection of endotoxin time zero. The rabbits were given periodic intravenous injections into an ear vein at −10 minutes, at time zero, and for every two hours thereafter for a 24 hour period. The results of this study can be seen in Table 8.

TABLE 8

EXPERIMENTAL ENDOTOXIN INDUCED SHOCK IN RABBITS; EFFECTS OF IL-1ra ON SURVIVAL RATE

	survival (no)					survival rate
	12 h	24 h	36 h	48 h	7 d	7 days (%)
A(N = 5)	5	5	5	5	5	100
B(N = 10)	9	6	3	2	2	20
C(N = 10)	9	7	4	3	2	20
D(N = 10)	10	7	6	5	4	40
E(N = 10)	10	10	10	9	9	90

In Table 8, Group A rabbits (n=5) were not given any endotoxin at time zero, and were given saline injections free of IL-1ra at the periodic injection times. Group B rabbits (n=10) were given 0.5 mg/kg of body weight of endotoxin at time zero, and the periodic injections were again free of IL-1ra. After 7 days the survival rate of rabbits in Group B was only 20%.

In Groups C–E (n=10), endotoxin was administered at time zero, and the saline injections contained varying amounts of IL-1ra. The rabbits in Group C received a total

of 10 mg/kg of body weight of IL-1ra. The rabbits in Group D received a total of 30 mg/kg of body weight of IL-1ra. And finally, the rabbits in Group E received a total of 100 mg/kg of body weight of IL-1ra. After 7 days, the survival rate of rabbits in Group E was 90%.

This experiment shows that treatment with IL-1ra significantly delays and reduces final mortality rates in rabbits with endotoxin induced shock.

EXAMPLE 8

Effects of Human IL-1ra on Ischemia and Reperfusion Injury

In the following example experimental dogs were subjected to regional myocardial ischemia for two hours and then reperfused for 4 hours. The dogs were divided into two groups, one group treated with IL-1ra and the other treated with serum albumin in the same buffer used for the test group.

Animals were fasted overnight and on the following morning, were anesthetized with 10 ml of thiamylal sodium 5%, followed by 2 ml of sodium pentobarbital 6%, intravenously. Additional sodium pentobarbital was administered during the experiment as necessary. Artificial respirator. A left thoracotomy was performed through the fifth intercostal space and polyvinyl catheters placed in the left internal jugular vein for fluid and drug administration, and in the left internal carotid artery and femoral arteries for pressure monitoring and withdrawal of reference blood samples. A catheter was placed in the left atrium for injection of radio active microspheres. The left circumflex artery was dissected free of surrounding tissue and an electromagnetic flow probe was placed on the vessel proximal to the first obtuse marginal branch. After an intravenous bolus injection of 50 mg of lidocaine, the circumflex coronary artery was occluded with the snare occluder for 2 hours. Complete occlusion was verified with the electromagnetic flow probe. The snare was then released suddenly, allowing reperfusion of the coronary vascular bed for 4 hours.

Two-dimensional echocardiograms and hemodynamic measurements (heart rate, blood pressure and left atrial pressure) was determined before occlusion, after 110 minutes of occlusion, 5 minutes after reperfusion, and 4 hours after reperfusion. Two-dimensional echocardiography was performed with the use of a scanner and a 2.25 MHz transducer. The transducer was placed on the closed shaved right chest and was allowed full visualization of the circumferential extent of the left ventricle in a short-axis projection. Echocardiographic images were recorded at the midpapillary muscle position onto a video cassette with use of a Sony recorder. A two dimensional echocardiographic analysis was performed with the use of a minicomputer-based video digitizing system.

End-diastolic and end-systolic frames were selected for analysis with the use of the onset of the Q wave in lead II as a marker of end-diastole and the smallest left ventricular cavity size as a marker of end-systole. Endocardial and epicardial borders for 3 consecutive beats during normal sinus rhythm was carefully traced directly from the video display onto a digitizing tablet. Quantitative analysis was performed with a radial contraction model and a fixed diastolic center of mass at 22.5 degree intervals over the full left ventricular circumference.

The midpoint of the posterior papillary muscle was chosen as a fixed anatomic reference and designated as 135 degrees. Wall thickening was computed for each of the 22.5 degree sectors with the following equation: wall thickening=[(end systolic wall thickness−end diastolic wall thickness)/end diastolic wall thickness]×100%. The normal range of wall thickening was determined from a functional map of the baseline images for three cardiac cycles and 95% tolerance limits were established in each animal. These limits were used for comparison with occlusion and reperfusion functional maps and abnormalities are expressed as the circumferential extent of dysfunction and the degree of dysfunction. The extent of dysfunction (in degrees) was measured at the intercepts between the occlusion or reperfusion maps and the lower 95% tolerance limit; the degree of dysfunction (in area units) is the planimetered area below the lower 95% tolerance limit.

Regional myocardial blood flow was assessed by the reference withdrawal method using tracer-tabled microspheres (15 μm diameter, New England Nuclear) injected into the left atrium. The microspheres were ultrasonicated and vortex-agitated before injection. Microspheres were injected before occlusion, after 110 minutes of occlusion, 5 minutes after reperfusion and 4 hours after reperfusion with one of six available isotopes (^{141}Ce, ^{51}Cr, ^{113}Sn, ^{103}Ru, ^{95}Nb, ^{46}Sc). Simultaneous reference arterial samples were withdrawn from the carotid and femoral arteries at a constant rate of 7 ml/minute with a Harvard withdrawal pump starting 10 seconds before microsphere injection and continuing for 120 seconds after completion of the injection.

Two adjacent transverse left ventricular slices at the midpapillary muscle level, corresponding to the echocardiographic short-axis slices, were selected for blood flow determination. Each slice was divided into 16 full-thickness 22.5 degree sectors. Each sector was then further divided into epicardial, midmyocardial, and endocardial samples. The tissue samples were then weighed, placed in counting vials, and assayed for radioactivity in a gamma scintillation counter. After background and overlap correction, absolute myocardial blood flow was calculated with the following equation: Qm=(Cm×Qr/Cr), where Qm=myocardial blood flow (ml/min); Cm=counts/min in tissue sample; Qr=withdrawal rate of the reference arterial sample (ml/min); Cr=counts/min in the reference arterial sample. Myocardial blood flow is expressed per gram of tissue for each sample.

Just prior to sacrifice, the left circumflex coronary artery was briefly occluded and monastral blue pigment (0.5 ml/kg) injected into the left atrium for delineation of the in vivo myocardial area at risk. The animal then received 3000 U of heparin and was sacrificed with an intravenous bolus of saturated KCI solution and the heart excised.

Treatment Groups. Dogs were randomly assigned to one of two groups. In the test group, dogs received a bolus injection of 30 mg IL-1ra just prior to the onset of the ischemia and 15 mg IL-1ra for each hour until the experiment was terminated. Control animals received an identical quantity of endotoxin-free, human albumin dissolved in the same buffer used for the test group.

Determination of Infarct Size. After death, the heart of each dog was excised, the left ventricle isolated from surrounding tissue, cooled in a freezer for 15 minutes, and then sliced into 5 mm transverse sections. The slices were then weighed and placed in a warm bath of buffered triphenyl tetrazolium chloride for ten minutes. In this technique, viable tissue stains red while nonviable tissue remains unstained (*Am. Heart J.*, 101:593). The unstained zone of infarcted tissue is outlined on transparent overlays and quantitated by planimetry using a microcomputer and cor-

rected for the weight of the heart slice. Infarct size is expressed as the percentage of the area of mycoardium at risk (the area at risk of infarction is defined as the area of the myocardium left unstained following the injection of monastral blue into the left atrium).

NMR Analysis of Myocardial Edema. After fixation, the hearts were cut into 5 to 7 transverse slices approximately 5 mm thick. Two transmural myocardial tissue samples were obtained from the nonischemic zone (positive monastral blue staining) and the central ischemic zone (negative blue staining). The epicardium for each sample was dissected away to eliminate possible lipid signal interference. Each piece was subdivided transmurally (weighing approximately 500 mg each) with one portion assessed for % H_2O by dessication technique (wet weight–dry weight/wet weight) while the other was placed into a clean dry glass tube. T1 and T1 relaxation times were obtained on a IBM PC 20 Minispec spectrometer (IBM Instruments, Inc., Danbury, Conn.) operating at 20 MHz and 40° C. The location of the sample in the magnet, 90° and 180° radio frequency pulses, and detector phase were optimized for each sample before relaxation measurements were obtained. T1 values were determined by a fit of 20 inversion data recovery points while T2 values were determined by using a Carr, Purcell, Meiboon-Gill (CPMG) sequence. In an attempt to minimize effects of diffusion and miscellaneous system instabilities, the 180° radio frequency interpulse spacing was maintained at 180 microseconds. The fraction of echo samples determined were used as variables to adjust the duration of the CPMG experiment. Typically, 1 to 150 data points were acquired as the echo train was delayed to 15% to 25% of its original amplitude. T2 values were determined by using a multi-exponential fit. Only the dominant component of the exponential fit was used for statistical analysis. The results of the T1 and T2 analysis were corrected with percent water for adjacent tissue samples to verify the accuracy of the NMR technique.

Histologic and Morphometric Evaluations. For each group tested at least 3 animals were evaluated by light microscopy. Sections stained with hematoxylin and eosin from each heart were evaluated for neutrophil accumulation within the area between viable and infarcted tissue.

Statistical Analysis. All data was represented as the mean±S.E.M. Comparisons within groups were made by a two-way analysis of variance. When significant F values are obtained, paired t tests (corrected for multiple comparisons with the Bonferroni inequality adjustment) will be used to determine which measurements differed significantly from one another.

Comparisons between groups were made by unpaired t test. An exponential regression was used to correlate infarct size data to myocardial blood flow.

The results of the IL-1ra treatment regimen on protecting dog myocardium from occlusion reperfusion injury, are listed in Table 9 below. As a percentage of the left ventricular mass, the influx infarct size in the treated group was reduced to 10.3% as opposed to 18.2% in the control animals. This result represents a 40% reduction in the percent of the left ventricular mass that was infarcted. The percentage of the area at risk, in contrast, was not markedly changed, 40.5% of the left ventricular mass in the treated group versus 44.8% in the control animals. When the infarcted area is calculated as a percent of the total area at risk, the numbers similarly favor the IL-1ra treated animals, 24.9% versus 42% in the control group.

TABLE 9

THE EFFECT OF IL-1ra IN REDUCING THE EXTENT AT INFARCTED TISSUE IN CANINE CORONARY OCCLUSION-REPERFUSION STUDIES

	IL-1ra Treated (n = 9)	Albumin Treated (n = 9)
Infarct size as a % of left ventricular mass	10.3% ± 2.2%	18.2% ± 3.3%
Area at risk as a % of left ventricular mass	40.5% ± 1.7%	44.8% ± 1.9%
Infarct size as a % of mass at risk	24.9% ± 4.6%	42% ± 8.3%

EXAMPLE 9

In Vivo Effects of IL-1ra on MBP-Induced EAE

Female Lewis rats (150–200 g) were purchased from Charles River Raleigh, N.C.), and housed at Synergen for at least 1 week before starting experiments. They received food and water ad libitum and housed in temperature and light controlled (12 h/day) rooms. Within each experiment, animals were age-matched.

EAE induction and evaluation. Rats (usually six/group) were anesthetized with 2% isoflurane+O_2 and immunized on day 0 in the footpad of the left hind limb with 0.1 ml of an emulsion containing MBP at one of the following doses 0, 3, or 30 μg (fragment 68–84 Bachem Bioscience, PA) dissolved in phosphate buffered saline (PBS) with an equal volume of complete Freund's adjuvant (CFA) containing 5 mg/ml of *Mycobacterium tuberculosis* H37Ra (Difco Lab MI). Control rats received 0.1 ml of the PBS/CFA emulsion with no MBP in the footpad of the left hind limb.

Evaluation of clinical disease was based on a conventional 0–5 scoring system. Briefly, the spectrum of rating was 0 normal, 0.5 partial loss of tail tone, 1 complete loss of tail tone, 2 dragging of one hind limb, 3 paralysis of both hind limbs, 4 morbid, and 5 death. Clinical severity was assessed on a daily basis. Daily weights were recorded for individual rats and weight loss/gain was expressed relative to initial weight.

The inhibitory effects of IL-1ra on clinical severity expressed as area under curve (Units arbitrary) were determined for each group and compared statistically against the vehicle group using the Mann-Whitney test. In addition to the clinical severity indices, mean weight gain for each group was determined and compared statistically (students t-test). In all of these studies no significant differences at any of the MBP doses were observed between the no vehicle and vehicle dosed groups.

The 3 and 30 μg MBP doses were used in the IL-1ra related studies, because the 3 μg and 30 μg doses were approximately submaximal and maximal for clinical symptoms without causing death in this model. All injections of IL-1ra (100 mg/kg) or diluent (CSE buffer: 10 mM citrate, 140 mM NaCl, 0.5 mM EDTA) control were given subcutaneously. IL-1ra or vehicle was administered every 6 hrs for 12 days beginning on day nine post MBP. In each experiment, the control rats received the same number of injections as the treatment groups to diminish any secondary effects due to stress.

Initial studies assessed the clinical severity of different doses of MBP (0.1–30 μg/0.1 ml) in the rat. The 0.1 and 0.3 μg MBP doses produced no apparent clinical symptoms. The 30 μg dose of MBP produced the most serve clinical

symptoms, compared to the 1 μg dose. This effect was highly significant ($p<0.001$, Mann-Whitney U-test). Onset of clinical symptoms varied from individual animal and on the concentration of MBP used, but significant differences between MBP doses were observed. In general, increasing the dose (1–30 μg) of MBP produced clinical symptoms earlier, for example 1 μg MBP had a mean±S.E.M. onset of 14.88±0.42 (n=9) compared to 12.35±0.16 (n=34; $p<0.01$) days for the 30 μg MBP dose. In addition, a dose dependent effect of MBP (1–30 μg) on weight loss was observed. Animals spontaneously recovered from the clinical symptoms within 5–7 days of onset. Administration of CFA alone produced no clinical symptoms, however, there was an initial transient weight loss compared to non-treated controls.

Effect of IL-1ra. To determine if inhibiting endogenous IL-1 in vivo would affect the development of EAE, daily (4× a day subcutaneously) injections of 100 mg/kg IL-1ra were initiated 9 days post MBP immunization and continued until day 21. IL-1ra significantly inhibited clinical symptoms induced by 3 ($p<0.032$) and 30 μg MBP ($p<0.001$) compared to vehicle controls. The IL-1ra treated groups exhibited a delay in the mean onset of clinical symptoms. For example, animals that received vehicle had a mean onset time of 13.8 and 11.5 days for the 3 or 30 μg MBP respectively, compared to the IL-1ra treated animals of 15.25 and 13.4 days, respectively.

At both doses of the MBP (3 μg or 30 μg) IL-1ra (100 mg/kg) decreased the duration of the clinical symptoms by 55% and 29% respectively compared to the vehicle groups (FIGS. **5** and **6**).

Weight loss is an important marker of EAE onset, and rats that received IL-1ra (100 mg/kg) lost less weight compared to the vehicle groups (FIG. **7**). The average weight gain for the 3 and 30 μg MBP groups that received vehicle over the 21 day study were 10±1.71 g and 4.6±2.2 g. The IL-1ra groups using similar doses of MBP (3 and 30 μg) had a significant weight gain compared to the vehicle groups 19±2.5 g and 17.6±0.42 g ($p<0.05$, $p<0.01$ unpaired t-test). The normal weight gain in unmanipulated animals over the same time period was 40±0.516 g.

The results implicate the involvement of endogenous IL-1 in EAE induction because treatment with the receptor antagonist (IL-1ra) reduced clinical disease. This most likely occurred by IL-1ra specifically binding to the IL-1 receptor complex thus preventing the binding of the natural endogenous ligand IL-1.

IL-1ra not only decreased the severity of the disease but there was also a trend in reducing EAE duration. Furthermore, the onset of the disease was also delayed by IL-1ra. Inhibition of weight loss due to IL-1ra treatment may have been secondary to suppression of EAE or due to blocking the ability of IL-1 to stimulate or synergize with TNF (Flores et al., 1989). It is also possible since IL-1ra reduced clinical symptoms, that animals found it easier to obtain food and water compared to their vehicle controls.

The IL-1ra treatment may have limited perivascular infiltration of cells into the CNS by preventing an IL-1-induced increase in adhesion molecule expression on the CNS vasculature. Astrocyte hyperplasia and gliosis result in the formation of CNS plaques associated with MS. As IL-1 has been shown to induce astrocyte proliferation, IL-1ra treatment may have prevented IL-1 induction of astrocyte hyperplasia. Finally, by interfering with the activity of IL-1, IL-1ra may have blocked the induction of other cytokines within the CNS that are involved in mediating the clinical and pathological sequela of EAE.

These studies have shown that selective inhibition of IL-1 by administering IL-1ra in vivo can reduce the clinical severity, duration and delay disease onset of the inflammatory CNS disease (EAE). Thus the IL-1 antagonist is therapeutically beneficial for certain inflammatory CNS diseases such as MS.

EXAMPLE 10

Effects of IL-1ra in Cerebral Ischemia

The objective of the experiments reported in this Example was to investigate the involvement of endogenous IL-1 in the damage following hypoxia-ischemia. To examine this hypothesis in neonatal brain IL-1ra was tested using an in vivo experimental rat model of hypoxic-ischemic forebrain injury. The neonatal brain hypoxic-ischemic model is widely recognized as a reliable and clinically relevant experimental procedure as a animal model of brain damage that can occur in cerebral palsy. Weighing the hemispheres has been shown to be a reliable method in quantifying tissue loss (Andine et al., *J. Neurosci. Meth.,* 35:253–260 (1990).

In the experiments, 7-day-old rats were used that had undergone a unilateral carotid ligation and subsequent exposure to 2 hours of 7.5% oxygen. This is a well characterized small animal model for perinatal hypoxic-ischemic encephalopathy (Rice et al., *Ann. Neurol.,* 9:131–141 (1981). This model, a variation of the preparation described in Levine, *Am. J. Pathol.,* 36:1–17 (1960) produces a unilateral brain injury, as described by Rice and colleagues. Neither unilateral carotid ligation nor hypoxia alone causes microscopically visible changes. Together, the two manipulations produce a spectrum of injury, varying according to the duration of hypoxia. The age of the rats were chosen because the development of rat brain at this stage resembles the maturational level of the full term human neonate. The pathogenesis of neuronal injury appears to be related to pressive ischemia that develops as the duration of hypoxic exposure lengths. The combination of moderate hypoxemia and ischemia causes a reproducible pattern of acute and chronic ischemic neuronal changes in the forebrain ipsilateral to carotid occlusion. Foci of infarction in the corpus striatum, hippocampus and cortex occur commonly, surrounded by areas of disrupted neuronal organization. The infarctions resemble the pattern of injury now being recognized more frequently in human infants as reported in Hill et al., *Pediatrics,* 71:790–93 (1983).

In the experiments, seven day old male and female (Sprague-Dawley) rats were anesthetized with 2% isoflurane+O_2 and body temperature was maintained within normal limits. The neck region was prepped with betadine solution before surgery. A small incision was made so that the left carotid artery was exposed. The carotid artery was electrocauterized between double ligatures of silk suture. The wound was closed with suture and the rats were placed back into a cage containing their dam to recover before they were placed into a chamber that contained either 20% or 7.5% O_2 in nitrogen.

After the rats had recovered from the anesthesia, they were placed into a chamber for 2 hours that was constantly gassed with either 7.5% (hypoxic environment) or 20% (normoxic environment) O_2 balanced nitrogen gas mixtures. The chamber was kept at 36° C. by placing it in a heated water bath. After this procedure the rats were returned to their dams.

IL-1ra (100 mg/kg subcutaneously) or vehicle (ml/kg subcutaneously) was administered either 1 hr prior to the

hypoxic chamber, or at time O, 1 or 3 hr post hypoxic insult and then at the following times 5, 9, 20 & 28 hrs.

Rats were sacrificed two weeks later and ipsilateral and contralateral brain hemisphere weights determined (wet weight) and then placed into a oven at 70° C. for 36 hrs (dry weight). Right and left hemisphere weight disparities were compared as percent reductions ([(L−R)/R]×100). During this phase of rapid brain growth, reduction in hemisphere weight can be used as an indicator of injury more readily than in adults. Hemispheric brain weight disparity, validated in this model as a quantitative measure of brain injury according to Andine et al., *J. Neurosci. Meth.*, 35:253–260 (1990), incorporated herein by reference, was determined.

In vehicle treated animals, the ischemic/hypoxic insult produced marked infarcts, the ipsilateral hemisphere being visually smaller in size and necrotic. In the vehicle treated animals the difference between the ipsilateral (0.5087±0.019 gms) and the contralateral (0.62±0.014 gms, n=19) hemispheres was approximately 120 mg, whereas the IL-1ra treated rats this difference was smaller 35 mg.

IL-1ra (100 mg/kg s.c.) administered prior or after the ischemic/hypoxic insult ameliorated the ischemic damage as measured by hemisphere weights. There was a reduction in the difference between the ipsilateral and contralateral hemisphere weights suggesting that IL-1ra was neuroprotective. IL-1ra was effective whether it was administrated 1 hour before or 1 hour after the ischemic/hypoxic insult.

When compared to vehicle treated hypoxic-ischemic controls, IL-1ra administered 1 hour prior to the ischemic-hypoxic insult and then after the ischemic-hypoxic insult at the time points indicated (1, 3, 5, 9, 20 and 28 hrs) was very effective in reducing cerebral hemisphere damage. Cerebral hemisphere weights in rats that underwent unilateral hypoxic-ischemic forebrain injury treated with either vehicle or IL-1ra 1 hr prior to the insult were determined as follows: vehicle treated: −20.84±4.2 (n=10); IL-1ra treated: −9.14±3.5 (n=10) ($p<0.05$). Littermate pups were treated with vehicle or IL-1ra immediately after the hypoxic-ischemic insult. The initial administration of IL-1ra at 0 hr after the insult was still effective in reducing cerebral hemisphere damage as shown by the following results: vehicle treated: −11.71±2.31 (n=16); IL-1ra treated: −0.168±2.50 (n=18) ($p<0.01$). Delaying the first administration of IL-1ra further to 1 hr post ischemic-hypoxic insult resulted in significant ($p<0.01$) reduction of cerebral hemisphere damage as shown by the following results: vehicle treated: −20.29±3.93 (n=20); IL-1ra treated: −5.16±2.0 (n=19). Further delays of the first dose of IL-1ra (3 hrs post insult) reduced the cerebral hemisphere damage as shown by the following results: vehicle treated: −12.78±3.74 (n=11); Il-1ra treated: −5.54±4.5 (n=11), but the reduction was not significantly different from vehicle treated animals.

Animals that did not undergo surgery and were placed in a normoxic (20% oxygen) environment, received IL-1ra or vehicle at the same dosing frequency as indicated above. These animals had normal hemisphere weights indicating that IL-1ra had no obvious effects on developing normal brain tissue.

In the present study, IL-1ra had little or no effect on normal brain tissue. In contrast, peripheral administration of IL-1ra reduced the damaged associated with the hypoxic-ischemic episode. It was possible in this model to delay the administration for up to 1 hr post insult and still retain significant neuroprotection. Further delay in the administration of IL-1ra was less effective, but there was still evidence of a neuroprotective role as indicated by the weight differences between the ischemic and non-ischemic hemispheres compared to vehicle treated animals. These data indicate that endogenous IL-1 is an important mediator of neuronal damage in this model since the IL-1 antagonist IL-1ra reduced the tissue damage.

The foregoing description of the invention is exemplary for purposes of illustration and explanation. It will be apparent to those skilled in the art that changes and modifications are possible without departing from the spirit and scope of the invention. It is intended that the following claims be interpreted to embrace all such changes and modifications.

What is claimed is:

1. A method for treating reperfusion injury comprising administering to a patient in need thereof a therapeutically effective amount of an interleukin-1 receptor antagonist (IL-1ra), wherein said IL-1ra comprises a polypeptide that inhibits IL-1 and is sufficiently pure such that at least a portion of the amino acid sequence of said polypeptide can be determined, wherein said polypeptide comprises

(1) the following amino acid sequence:

(U) Arg Pro Ser Gly Arg Lys Ser Ser Lys Met Gln Ala Phe Arg Ile Trp Asp Val Asn Gln Lys Thr Phe Tyr Leu Arg Asn Asn Gln Leu Val Ala Gly Tyr Leu Gln Gly Pro Asn Val Asn Leu Glu Glu Lys Ile Asp Val Val Pro Ile Glu Pro His Ala Leu Phe Leu Gly Ile His Gly Gly Lys Met Cys Leu Ser Cys Val Lys Ser Gly Asp Glu Thr Arg Leu Gln Leu Glu Ala Val Asn Ile Thr Asp Leu Ser Glu Asn Arg Lys Gln Asp Lys Arg Phe Ala Phe Ile Arg Ser Asp Ser Gly Pro Thr Thr Ser Phe Glu Ser Ala Ala Cys Pro Gly Trp Phe Leu Cys Thr Ala Met Glu Ala Asp Gln Pro Val Ser Leu Thr Asn Met Pro Asp Glu Gly Val Met Val Thr Lys Phe Tyr Phe Gln Glu Asp Glu

wherein (U) is nothing or Met; or

(2) a sequence which is at least about 90% homologous to said amino acid sequence.

2. The method of claim **1**, wherein said IL-1ra is glycosylated.

3. The method of claim **1**, wherein said IL-1ra is non-glycosylated.

4. The method of claim **3**, wherein said IL-1ra is methionyl IL-1ra.

5. The method of claim **1**, wherein said IL-1ra is produced by recombinant DNA methods.

6. A method according to claim **1**, wherein said polypeptide comprises the following amino acid sequence:

(U) Arg Pro Ser Gly Arg Lys Ser Ser Lys Met Gln Ala Phe Arg Ile Trp Asp Val Asn Gln Lys Thr Phe Tyr Leu Arg Asn Asn Gln Leu Val Ala Gly Tyr Leu Gln Gly Pro Asn Val Asn Leu Glu Glu Lys Ile Asp Val Val Pro Ile Glu Pro His Ala Leu Phe Leu Gly Ile His Gly Gly Lys Met Cys Leu Ser Cys Val Lys Ser Gly Asp Glu Thr Arg Leu Gln Leu Glu Ala Val Asn Ile Thr Asp Leu Ser Glu Asn Arg Lys Gln Asp Lys Arg Phe Ala Phe Ile Arg Ser Asp Ser Gly Pro Thr Thr Ser Phe Glu Ser Ala Ala Cys Pro Gly Trp Phe Leu Cys Thr Ala Met Glu Ala Asp Gln Pro Val Ser Leu Thr Asn Met Pro Asp Glu Gly Val Met Val Thr Lys Phe Tyr Phe Gln Glu Asp Glu

wherein (U) is nothing or Met.

7. The method according to claim **6**, wherein the polypeptide is substantially pure.

8. The method according to claim **1**, wherein the IL-1ra is IL-1rax, IL-1raα or IL-1raβ.

9. The method according to claim **1**, wherein said polypeptide is linked to a polymeric material.

10. The method of claim **9**, wherein the polymeric material is polyethylene glycol.

11. The method of claim 1, 9, or 10, wherein the polypeptide is administered in a pharmaceutical composition.

12. The method according to claim 6, wherein said polypeptide is linked to a polymeric material.

13. The method of claim 12, wherein the polymeric material is polyethylene glycol.

14. The method of claim 6, 12 or 13, wherein the polypeptide is administered in a pharmaceutical composition.

15. The method of claim 11, wherein said pharmaceutical composition comprises the polypeptide and a carrier, wherein said composition is a pharmacologically-compatible, slow-release formulation.

16. The method of claim 14, wherein said pharmaceutical composition comprises the polypeptide and a carrier, wherein said composition is a pharmacologically-compatible, slow-release formulation.

17. The method of claim 6, wherein said polypeptide consists of the following

(U) Arg Pro Ser Gly Arg Lys Ser Ser Lys Met Gln Ala Phe Arg Ile Trp Asp Val Asn Gln Lys Thr Phe Tyr Leu Arg Asn Asn Gln Leu Val Ala Gly Tyr Leu Gln Gly Pro Asn Val Asn Leu Glu Glu Lys Ile Asp Val Val Pro Ile Glu Pro His Ala Leu Phe Leu Gly Ile His Gly Gly Lys Met Cys Leu Ser Cys Val Lys Ser Gly Asp Glu Thr Arg Leu Gln Leu Glu Ala Val Asn Ile Thr Asp Leu Ser Glu Asn Arg Lys Gln Asp Lys Arg Phe Ala Phe Ile Arg Ser Asp Ser Gly Pro Thr Thr Ser Phe Glu Ser Ala Ala Cys Pro Gly Trp Phe Leu Cys Thr Ala Met Glu Ala Asp Gln Pro Val Ser Leu Thr Asn Met Pro Asp Glu Gly Val Met Val Thr Lys Phe Tyr Phe Gln Glu Asp Glu

wherein (U) is nothing or Met.

18. The method of claim 17, wherein the polypeptide is administered in a pharmaceutical composition.

19. The method of claim 18, wherein the pharmaceutical composition comprises the polypeptide and a carrier, wherein the composition is a pharmacologically-compatible, slow-release formulation.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,159,460
DATED : December 12, 2000
INVENTOR(S) : Thompson et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [63], line 11, insert after "abandoned" the following:
-- which is a continuation of application No. 07/248,521, September 23, 1988, abandoned, which is a continuation-in-part of application No. 07/238,713, August 31, 1988, abandoned, which is a continuation-in-part of application No. 07/199,915, May 27, 1988, abandoned --

Column 11,
Line 13, delete "is".
Line 45, change "Saklatvala" to -- Saklatavala --.
Line 63, change "Theumatol." to -- Rheumatol. --.

Column 20,
Table 7, line 4, change "0.8" to -- 0/8 --.

Column 29,
Line 19, insert after "following", -- amino acid sequence: --.

Signed and Sealed this

Eighteenth Day of November, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,159,460
DATED : December 12, 2000
INVENTOR(S) : Thompson et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [63], line 11, insert after "abandoned" the following:
-- which is a continuation of application No. 07/248,521, September 23, 1988, abandoned, which is a continuation-in-part of application No. 07/238,713, August 31, 1988, abandoned, which is a continuation-in-part of application No. 07/199,915, May 27, 1988, abandoned --

Column 11,
Line 13, delete "is".
Line 45, change "Saklatvala" to -- Saklatavala --.
Line 63, change "Theumatol." to -- Rheumatol. --.

Column 20,
Table 7, line 4, change "0.8" to -- 0/8 --.

Column 29,
Line 19, insert after "following", -- amino acid sequence: --.

Signed and Sealed this

Eighteenth Day of November, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office